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Association of biomaterials and mesenchymal stem cells from Wharton's jelly of human umbilical cord for promotion of peripheral nerve regeneration:

A functional and morphological study.

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A functional and morphological study.

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DECLARATION:

The author declares that in accordance with "*nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009"* she has managed the majority of design and technical execution of the work and results interpretation came out with the following published and submitted articles:

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Articles as co-author that complement the introduction and discussion of these work.

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"Challenges are what make life interesting and overcoming them is what makes life meaningful."

Joshua J. Marine

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Abbreviations

BDNF - Brain-derived neurotrophic factor

cAMP - Adenosine 3’;5’-cyclic monophosphate

CB - Cord Blood

CE - European Conformity

CNF - Ciliary neurotrophic factor

CNS - Central Nervous System

DMSO - Dimethylsul-foxide

DMEM - Dulbecco's Modified Eagle Medium

DPBS - Dulbecco's Phosphate-Buffered Saline

ECM - Extracellular Matrix

EPT - Extensor Postural Trust

FBS - Fetal Bovine Serum

FDA - Food and Drug Administration

GAP - 43 - Anti-growth associated protein-43

GDNF - Glial cell line-derived neurotrophic factor

GFAP - Anti-glial fibrillary acidic protein

GPTMS - γ-glycidoxypropyltrimethoxysilane

HBSS - Hank's Balanced Salt Solution

HLA - Human Leucocyte Antigen

HMSCs - Human Mesenchymal Stem Cells

IGF-1 - Insulin-like growth factor-1

MNS - Motor Nervous System

MSCs - Mesenchymal Stem Cells

NCs - Nerve Conduits

NeuN - Anti-neuronal nuclei

NGF – Nerve Growth Factor

PLA - Poly(L-lactide)

PLC - Poly(DL-lactide-ε-caprolactone)

PLG - Poly(glycolide)

PLGA - Poly(lactic-co-glycolic acid)

PNI - Peripheral Nerve Injuries

PNS - Peripheral Nervous System

PVA - Polyvinil Alcohol hydrogel

SCs - Schwann Cells

SEM - Scanning Electron Microscope

SFI - Sciatic Functional Index

SNS - Sensory Nervous System

SSI - Sciatic Static Index

StC - Stem Cells

UC - Umbilical Cord

UCX® - Human Stromal Cells from Wharton's jelly of Umbilical Cord Tissue

WRL - Withdrawal Reflex Latency

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Resumo

Com o aumento da esperança média de vida são várias as doenças incapacitantes que surgem no envelhecimento e com grande impacto na qualidade de vida do Homem. Podemos incluir nesta categoria as doenças cardiovasculares, neurológicas, musculosqueléticas e várias outras doenças de origem maligna. Daí a urgência e importância de desenvolver novos tratamentos mais eficientes e capazes de minimizar ou até eliminar estas incapacidades. A seguir às lesões do sistema nervoso central (SNC), as lesões do sistema nervoso periférico (SNP) são as que têm menor sucesso no que respeita à recuperação funcional apesar da melhoria constante das técnicas de microcirurgia surgirem com elevada frequência na prática clínica. Uma total compreensão quer molecular quer celular, do processo regenerativo do nervo periférico incluindo da recuperação funcional e de uma (possível) reinervação do(s) órgão(s) alvo, após uma lesão de axonotmese ou de neurotmese, continua a ser um dos principais objectivos da biologia e medicina regenerativa. O modelo experimental *in vivo* mais utilizado em estudos de regeneração do nervo periférico tem sido o nervo ciático do rato. Apesar das lesões do nervo ciático não serem comuns em Humanos, este modelo experimental é muito realista no que respeita ao estudo de nervos mistos ou plurifasciculares com axónios de diferentes tamanhos e origens. Nos últimos anos, os avanços na Medicina Regenerativa têm sido notáveis, e a comunidade científica tem testemunhado a descoberta de novos conceitos com grande impacto na área. Até há alguns anos atrás era do entendimento geral que a maioria dos tecidos apresentava uma capacidade limitada de regeneração. Hoje em dia é do conhecimento comum que quase todos os órgãos e tecidos poderão regenerar, parcial ou totalmente (num cenário ideal) com base em terapias celulares e engenharia de tecidos. No que se refere à engenharia de tecidos e às terapias celulares, as células estaminais mesenquimatosas (MCSs) têm suscitado bastante interesse devido às suas características, nomeadamente, elevada plasticidade, capacidade de proliferação e diferenciação, assim como as suas propriedades imunossupressoras. As MSCs têm a capacidade de produção de factores tróficos, produção de moléculas da matriz extracelular, modulação da resposta imunológica e de diferenciação em células de Schwann ou noutras células envolvidas na degenerescência Walleriana e regeneração axonal. Estudos recentes demonstram que as MSCs são capazes de modificar o microambiente em torno do tecido lesionado, promovendo a reparação com melhorias funcionais através da secreção de factores de crescimento, conhecido como o efeito paracrino, que estimulam as populações de células existentes no local, diminuindo a resposta imune e inflamatória. Estas células têm ganho adeptos

devido a vários fatores: i) maior número de células estaminais por volume; ii) não é necessária uma compatibilidade antigénica HLA tão exigente como para as células da medula óssea, alargando o número de dadores disponíveis, r baixa expressão dos antigénios HLA classe I e ausência de expressão dos antigénios HLA classe II; e iii) são mais fáceis de obter, processar e criopreservar e a sua colheita é eticamente aprovada e legislada pelas leis nacionais e internacionais.

No presente trabalho pretendeu-se avaliar o efeito terapêutico das MSCs humanas (HMSCs), isoladas da geleia de Wharton do cordão umbilical (UC), em lesões de axonotmese e neurotmese do nervo ciático do rato. Este manuscrito inclui os resultados obtidos com HMSCs indiferenciadas e diferenciadas em células tipo neurogliais, infiltradas localmente na lesão ou associadas a diferentes biomateriais, dois dos quais já disponíveis no mercado: poly(DL-lactide-ε-caprolactone) (PLC, Vivosorb®), um híbrido de quitosano e um selante hemostático composto por colagénio e trombina (Floseal®). Uma vez que estes biomateriais já foram previamente estudados pelo nosso grupo de investigação em lesões de axonotmese e de neurotmese do nervo ciático, este trabalho focou-se no desenvolvimento de um sistema celular seguro e capaz de expansão *in vitro* – as MSCs isoladas da geleia de Wharton do UC. Os estudos *in vitro* das HMSCs indiferenciadas e sujeitas a diferenciação, incluíram avaliação de citocompatibilidade dos biomateriais desenvolvidos, caracterização celular por imunocitoquímica, análise de cariótipo, citometria de fluxo e perfil metabólico durante a expansão e a diferenciação. Os ensaios *in vivo* recorrendo a lesões de axonotmese e de neurotmese do nervo ciático de ratos adultos *Sasco Sprague*, incluíram uma análise em termos de biocompatibilidade, recuperação funcional durante 12 ou 20 semanas respectivamente e análise morfológica por histomorfometria. As HMSCs isoladas da geleia de Wharton do UC associadas aos diversos biomateriais utilizados, são uma potencial ferramenta para promover a recuperação funcional e morfológica, em especial, de nervos sensitivos após traumatismo, especialmente em lesões de neurotmese quando não é possível realizar uma sutura epineural topo-a-topo sem tensão ou um autoenxerto. Estas células não apresentam qualquer controvérsia ética, podem ser colhidas no momento do parto, criopreservadas, descongeladas e expandidas para aplicações terapêuticas. Da necessidade de um estudo longitudinal e do envolvimento multidisciplinar incluindo o desenvolvimento de biomateriais e de sistemas celulares, bem como para ensaios pré-clínicos recorrendo a modelos animais apropriados, o bem-estar animal e a legislação em vigor foram sempre considerados desde o primeiro dia deste trabalho experimental.

Abstract

With the world wide global increase in life expectancy, a variety of disabling diseases with large impact on human population are arising. This includes cardiovascular, neurological, musculoskeletal, and several other malignancies. Therefore, it is imperative that new and more effective treatment procedures are developed to minimize or even rectify these changes. After Central Nervous System (CNS) lesions, Peripheral Nervous System (PNS) injuries are the ones with minor successes in terms of functional recovery and the most frequent in clinical practice. A full understanding of peripheral nerve regeneration process, especially what concerns a complete functional recovery and organ reinnervation after nerve injury, still remains the principle goal of regenerative biology and medicine. The rat sciatic nerve has been the most commonly experimental animal model used in studies concerning peripheral nerve regeneration and possible therapeutic approaches. Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets. Recent advances in Regenerative Medicine are remarkable and in the last years the scientific community has witnessed the arrival of many new concepts and discoveries. Until a few years ago, biological tissues were regarded as unable of extensive regeneration, but nowadays organs and tissues appear as capable to be reconstructed, based on “stem cells”. Mesenchymal stem cells (MSCs) have become one of the most interesting targets for tissue regeneration due to their high plasticity, proliferative and differentiation capacity together with their attractive immunosuppressive properties. Cellular systems implanted into an injured nerve may produce growth factors or extracellular matrix molecules, modulate the inflammatory process and eventually improve nerve regeneration. It has been speculated that once MSCs have the potential to differentiate into several tissues, they might be responsible for turnover and maintenance of adult tissues. Recent studies demonstrated that transplanted MSCs are able of modifying the surrounding tissue microenvironment, promoting repair with functional improvement by secretion of growth factors (known as paracrine effect), by stimulating the pre-existing stem cell populations and by decreasing the inflammatory and immune response. The stem cells obtained from the umbilical cord are a promising alternative once: i) the number per volume is higher, ii) a complete or high HLA profile match for allogenic use is not necessary due to the low expression of HLA class I antigens in addition to the absence of HLA class II expression, which permits a great enlargement of the number of available donors iii) the stem cells are

easier to obtain, and to be cryopreserved, and their collection is ethically approved by national and international laws.

In the present study, the therapeutic value of human umbilical cord matrix (Wharton's jelly) MSCs (HMSCs) on rat sciatic nerve after axonotmesis and neurotmesis injuries, was evaluated. This manuscript includes the results obtained with undifferentiated HMSCs or differentiated into neuroglial-like cells locally infiltrated or delivered by different biomaterials, two of them already available in the market: poly(DL-lactide-ε-caprolactone) (PLC Vivosorb®) membrane, hybrid chitosan developed by our research group, and a haemostatic sealant composed of collagen-derived particles and topical bovine-derived thrombin (Floseal®). Since these biomaterials were already previously tested by our research group in sciatic nerve injuries, this work was focused in the development of a safe and *in vitro* expandable cellular system – the MSCs from the Wharton's jelly of the umbilical cord. The *in vitro* studies of the scaffolds included cytocompatibility evaluation of the biomaterials used and cell characterization by immunocytochemistry, karyotype analysis, differentiation capacity into neuroglial-like cells, metabolic profile during expansion and differentiation, and flow cytometry. In the present work MSCs were expanded and differentiated *in vitro* in neuroglial-like cells and *in vivo* testing was carried out in *Sasco Sprague* adult rats. After axonotmesis and neurotmesis injuries and therapeutic treatment, follow-up for a complete functional evaluation was performed during 12 to 20 weeks, respectively and the repaired nerves processed for stereological analysis, allowed a morphologic regeneration evaluation. The MSCs from the Wharton's jelly delivered through tested biomaterials should be regarded as a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves when an epineural end-to-end suture without tension or grafting are not possible. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses. The importance of a longitudinal study concerning tissue engineering of the peripheral nerve, which includes a multidisciplinary team able to develop biomaterials associated to cell therapies, to perform pre-clinical trials concerning animal welfare and the appropriate animal model was always enhanced since the first day of this experimental work.

Chapter I

Introduction

Unless otherwise indicated, drawings included in this chapter were created by Hanna Gärtner, 2013.

Introduction

Peripheral neuropathy may be inherited or acquired. The inherited are caused by inborn mutations. Some genetic mistakes lead to mild neuropathies with little significant impairment, starting at early adulthood. Other genetic alterations are more severe and frequently appear in infancy or childhood and are known as disorders that are responsible for extreme weakening and wasting of muscles in the lower legs and feet, gait abnormalities, loss of tendon reflexes, and numbness in the lower limbs, among other symptoms. Acquired peripheral neuropathies are grouped into three broad categories: (i) caused by systemic disease; (ii) caused by trauma from external agents, and (iii) caused by infections or autoimmune disorders affecting nerve tissue. It is recurrent that the specific cause is unknown and sometimes iatrogenic. Clinicians usually refer to neuropathies with no known cause as idiopathic neuropathies. This thesis is focused in peripheral nerve injuries caused by traumatic/physical injuries, which are the most common. Sudden trauma, such as motor vehicle accidents, falls, industrial accidents, penetrating traumas and sport-related activities cause severe lesions to nerves.

Even after many years of research, results are very disappointing specially concerning the functional recovery of the peripheral nerve (Flores *et al.,* 2000). It is crucial to have a complete knowledge of peripheral nerve anatomy before we start any kind of study. The perfect knowledge passes through a solid understanding of the various components of intraneural arrangements (Flores *et al*., 2000). Before a deep dive in the peripheral nerve regeneration it is vital to have an overview on the anatomy and physiology of the nervous system especially on the peripheral nervous system (PNS).

1. Nerve Anatomy

The nervous system coordinates internal organ functions and responds to external environmental stimulus. It is divided into the central nervous system (CNS) and the peripheral nervous system (PNS); the last was intensively studied for the past years and the basis of the research work of the PhD thesis, described in this manuscript. The PNS functions in order to receive and respond to stimuli for and from the brain and spinal cord which together form the CNS (McKinley *et al.*, 2008). The cranial nerve, spinal nerve, plexuses, ganglia and peripheral nerves all together constitute de PNS (McKinley *et al.*, 2008).

The CNS and PNS together have three main functions: collecting information (receptors - PNS), processing and evaluating information (sensory system – PNS and CNS) and

responding to stimuli (motor system – PNS and CNS) (McKinley *et al*., 2008). Mainly we have two functional divisions for nervous system: sensory nervous system (SNS) and motor nervous system (MNS).

In SNS nerves from PNS diffuse sensory information to specific parts of brain and spinal cord and the CNS interprets the information, so is responsible for input. There are two components in this system, the somatic sensory and the visceral sensory component. The somatic is considered a voluntary mechanism once there is some kind of control on it; this includes the somatic and special sense. The visceral is considered involuntary because there is no control over it (McKinley *et al.*, 2008). On the other hand, the CNS is responsible for the output, so transmitting motor stimuli from de CNS to muscles and glands. The peripheral nerve organization has been studied for at least two centuries (Stewart, 2003) as described.

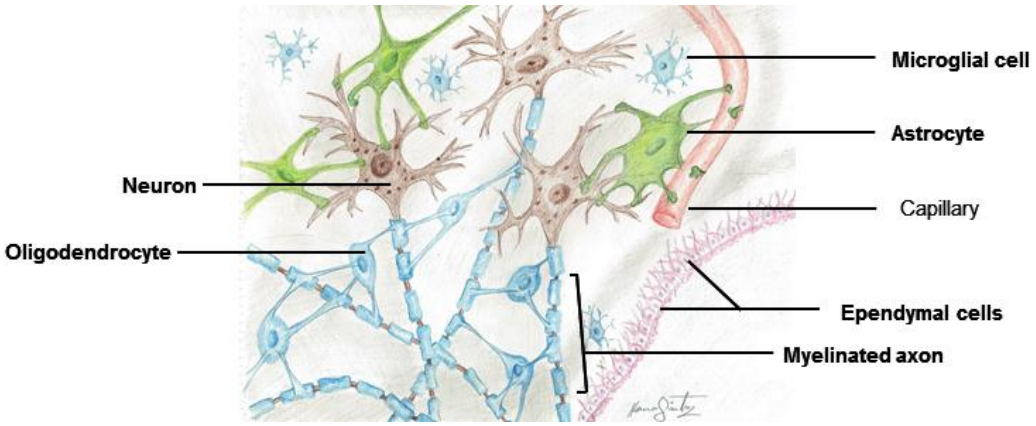

Figure 1 – Cellular organization within nervous tissue.

1.1. Neurons

Neurons are the basic functional unit of nervous system. They have a high metabolic rate, survival depending on a continuous and abundant supply of glucose and oxygen. They have an extensive longevity and may survive since fetal development until elder age. They are unable to divide, typically are considered as unmitotic (McKinley *et al.,* 2008).

Although neurons may appear in many shapes and sizes, they all consist of the following common basic structures (Figure 2):

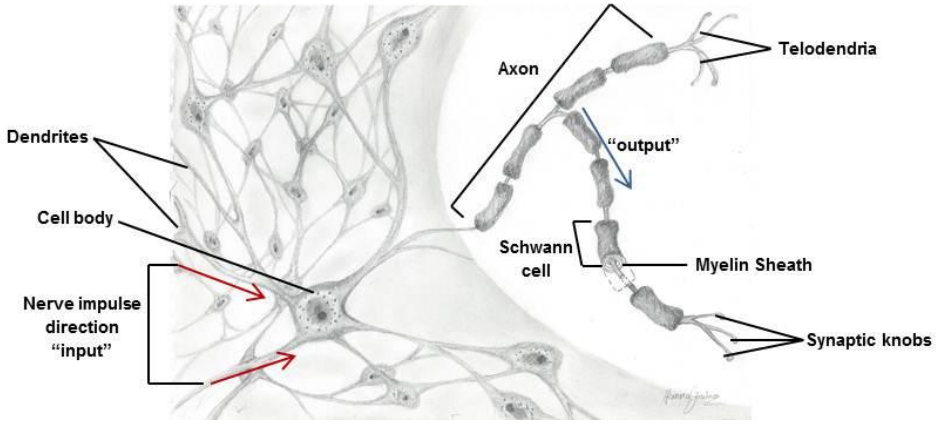

Figure 2 – Representation of neuron structure.

- Cell body – or the soma is the control centre, being mainly responsible for receiving, transforming and redirecting the nerve impulse. Surrounded by a plasma membrane the cell body encloses a nucleus where the high metabolic activity occurs.
- Dendrites – short branching fibres from the cell body. There may be one or more for each neuron. The more dendrites a neuron has, higher is the contact surface with the surroundings which permits higher input of nerve impulses. This structure is essentially responsible for the input of the nerve impulse.
- Axon – elongated projection from the cell body, also named nerve fibre. A neuron may have one or no axon at all. They are responsible for the output of information.

Neurons may be classified according to their morphological structure (table 1) or function (Table 2). The structural classification depends on the number of dendrites extending from the cell body.

Table 1: Structural classification of neurons

Classification	Description	Function
Unipolar Neuron	No dendrites only with a single axon, normally with a T shape resulting from the fusion of two processes into one long axon	Sensory neuron
Bipolar Neuron	Very uncommon with one dendrite and one axon	Special sensor neurons found in retina and olfactory epithelium
Multipolar Neuron	Most common with several dendrites and mostly one axon	Interneurons and motor neurons

Adapted from (McKinley *et al*., 2008)

Table 2: Functional Classification of Neurons

Classification	Description	Structural
Sensory	Conducts nerve impulse from body to CNS	Mostly unipolar and a few bipolar
Motor	Conducts nerve impulse from CNS to muscles and glands	Multipolar
Interneuron	Existing only in CNS; facilitating communication between motor and sensory neurons	Multipolar

Adapted from (McKinley *et al*., 2008)

Considering function, neurons may be classified as sensory, motor or interneurons. As described on table 2.

1.2. Glial Cells

Glial cells differ from neurons in size, since these are smaller cells, and on mitotic capacity, once they are able to divide (McKinley *et al*., 2008). There are two types of PNS glial cells, the satellite flat cells arranged around neural cell bodies in ganglia and the neurolemmocytes called Schwann cells (SCs), associated with PNS neurons and responsible for myelinating PNS axons and for surrounding the unmyelinated axons. Peripheral nerve regeneration is entirely dependent on the SCs, which play a vital role in the guidance of axonal growth towards distal end during regenerative process after axonotmesis and neurotmesis injuries. They are also responsible for generating an appropriate environment in the injured tissue as they produce extracellular matrix (ECM), cell adhesion molecules, integrins and neurotrophins and play a fundamental role during the Wallerian degeneration (Schmidt *et al.*, 2003).

1.3. Peripheral Nerve System

The peripheral nerve (Figure 3) is composed of several axons with various sizes which are surrounded by connective tissue and nerve components. The outer layer of connective tissue is the epineurium that drags all fascicles together, this is the stronger sheath. The perineurium surrounds one or more fascicles that for its turn contain individual axons and SCs. The endoneurium is the last sheath that surrounds the myelinated axon or unmyelinated cluster of nerve fibres associated with SCs. All together

form a network that organize and protects the fibres and axons (Flores *et al*., 2000; Eroschenko, 2012).

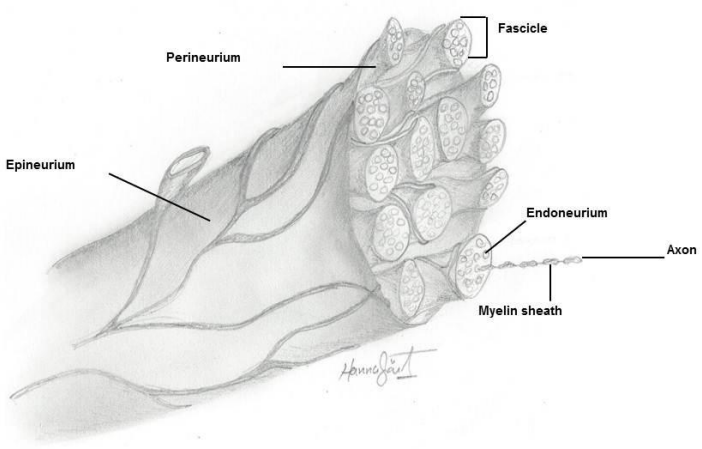

Figure 3 – Cross section of peripheral nerve

The number and size of fascicles diverge significantly, not only among different nerves but also inside the same nerve in different sites along the structure (Stewart, 2003). Nerves can be mono, oligo and poly-fascicular. About longitudinal organization of these fascicules, two opposite hypothesis have arisen in these last two centuries. The first one defines that the fascicules are settled like cables (Figure 4A) and the more recent views is that along the structure there is branching, splitting and re-joining of fascicules and parts of it, the so called plexiform structure (Figure 4B) (Stewart, 2003). Evidence has shown that in truth there is a mixture of both theories.

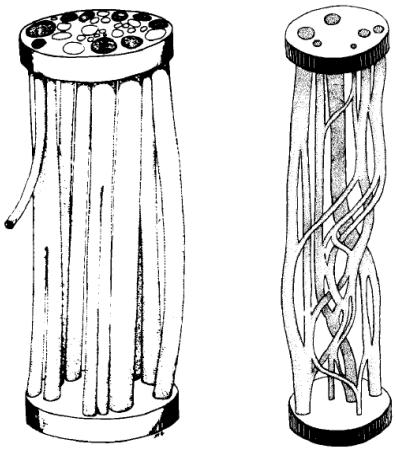

Figure 4 - Representation of the two hypotheses for fascicular organization of peripheral nerve. (A) Cable structure and (B) Plexiform (Stewart, 2003).

2. Nerve Injury

Many people are affected by peripheral nerve injuries (PNI) every year, striking about 2.8% of trauma patients (Deumens *et al*., 2010b). A large number of patients remain with the incapacity for all their life's (Madduri *et al*., 2010), this brings huge disabilities across the world. These injuries are of extreme importance since they lead to disturbances in functions mediated by the injured nerve (Deumens *et al*., 2010b) and there for the delay, or even preclude the return to work of the patients. PNI also compromise the quality of life of patients as it increases the risk of secondary disabilities resulting from falls and secondary injuries (Robinson, 2000). Until late 18th century, it was believed that peripheral nerves would never regenerate (Siemionow *et al*., 2010). Since then, many advances have been done in this field, but it still remains a clinical challenge.

2.1. Classification

Peripheral nerve injury may present or not gaps between nerve stamps. Axons extensions can regenerate over the gap reconnecting to distal stump (Siemionow *et al.*, 2010). In the absence of an intervention, functional regeneration is restricted due to scars, neuroma formation, mismatched fibres or extensive splitting of the re-growing axons; moreover peripheral regeneration is often associated to neuropathic pain, which is even more limiting for the patient than the incomplete functional recovery (Keilhoff *et al.*, 2011). The functional outcome gets worse with the degree of injury and regeneration is weaker if a nerve gap or neuroma has been developed leading to total functional impairment (Siemionow *et al.*, 2010; Keilhoff *et al.*, 2011). Temporal delay from time of trauma to surgical repair is also an important factor for poor functional outcome (Walsh *et al.*, 2009). There are two proposed classification for peripheral nerve injuries (Table 3). One proposed by Seddon in 1975 and another proposed by Sunderland in 1978 (Robinson, 2000). The Seddon classification is more commonly used in literature and among the clinical field. The one proposed by Sunderland is a more subdivided scheme and less straight forward (Robinson, 2000).

As described by Seddon, neuropraxia is a minor injury with motor and sensory loss but with no evidence of Wallerian degeneration (Robinson, 2000). Axonotmesis lesion frequent in crush, nerve stretch and percussion injuries. The axon and myelin sheaths disrupt but the surrounding stroma maintains partially or even totally intact. Wallerian degeneration occurs but axonal regeneration can follow along the intact endoneurial tube (Robinson, 2000). Resuming, neuropraxia and axonotmesis have a good clinical

prognosis and a surgical reconstruction is not need in most of the cases. Neurotmesis is an injury where the nerve is completely transected, with or without loss of tissue (gap) and it may be evidently disorganized by scars, without a surgical reconstruction the axonal regrowth is impossible (Robinson, 2000). Normally it is associated with an evident poor functional recovery comparing with other injuries, especially if the distance between injury and target organ or tissue is quite long (Walsh *et al.*, 2009).

Table 3: Classification system for nerve injuries

Seddon Classification	Sunderland Classification	Pathology	Prognosis
Neuropraxia	First degree	Myelin injury or ischemia	Excellent recovery in weeks to months
Axonotmesis		Axon loss Variable stromal disruption	Good to poor, depending upon integrity of supporting structures and distance muscle
	Second degree	Axon loss Endoneurial tubes intact Perineurium intact Epineurium intact	Good, depending upon distance muscle
	Third degree	Axon loss Endoneurial tubes disrupted Perineurium intact Epineurium intact	Poor Axonal misdirection Surgery may be required
	Fourth degree	Axon loss Endoneurial tubes disrupted Perineurium disrupted Epineurium intact	Poor Axonal misdirection Surgery usually required
Neurotmesis	Fifth degree	Axon loss Endoneurial tubes severed Perineurium severed Epineurium severed	No spontaneous recovery Surgery required Prognosis after surgery reserved

Adapted from (Robinson, 2000)

2.2. Wallerian Degeneration

PNS axons are very vulnerable to traumas like cross section and crushing. However, regeneration of axons is possible depending on the extension of the lesion. It depends on 3 factors: (i) the amount of damage; (ii) the secretion of growth factors and (iii) the

distance between site of injury and the effector organ. This regeneration process is called Wallerian degeneration.

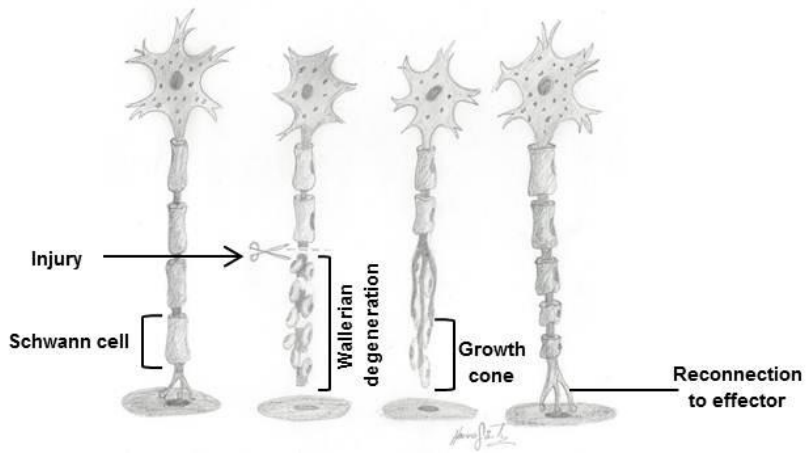

Figure 5 - After axonotmesis a traumatic degeneration occurs in the proximal nerve segment and the immediate distal portion of the nerve. Within 2 to 4 days, Wallerian degeneration occurs in the distal segment of the axon and lasts for 1 to 2 weeks.

After a trauma peripheral nerve regeneration is basically supported by SCs (Keilhoff *et al.*, 2011), who play a vital role during the Wallerian degeneration (Figure 5) in which the distal segment suffers a slow process of degradation. The SCs are responsible for removing the debris, which is the primordial step of this process which starts immediately after injury and implicates myelin breakdown. The end of the proximal portion seals off by membrane fusion and swells. This is a result of cytoplasm flowing from the neuron cell body through the axon. The distal portion of the axon and its myelin sheath degenerate, SCs and macrophages are recruited to the damaged site in order to phagocyte all myelin and cellular debris. These SCs organize themselves into the regeneration columns, called the Büngner bands, which guide the axon development as it begins to grow rapidly through the regeneration tube at a rate of about 5 mm per day under the influence of nerve growth factors released by the SCs. Following Wallerian degeneration the axon regenerates and remyelination occurs. Innervation is restored as soon as the axons re-established contact with its original effector organ or tissue (Flores *et al.*, 2000; McKinley *et al.*, 2008; Keilhoff *et al.*, 2011).

Proliferating SCs produce neurotrophins, cell adhesion molecules, and cytokines which support axonal regeneration and recruit more cells into the injured site (Walsh *et al.*, 2009). Axonal regeneration depends on SCs promotion, so unless nerve contact is rebuilt quickly when there is a gap between the nerve stumps, SCs lose their ability to assist

regeneration which leads to a poor functional recovery and in most cases, to neuroma formation (Keilhoff *et al.*, 2011).

3. Surgical reconstruction of peripheral nerve injuries

3.1. Microsurgical procedure

Reconstructive repair strategies are based on nerve injuries from 3rd degree forward, on the other hand, 1st and 2nd degree lesions are left to heal spontaneously (Pfister *et al.*, 2011). At this moment surgery is the only treatment for severe injured nerves, when the gap is short and a direct tension-free suture coaptation of the nerve stumps is possible (Madduri *et al.*, 2010).

The rat is the most commonly used animal model for peripheral nerve regeneration studies, due to both its widespread availability and the distribution of their nerve trunks which is similar to humans (Mackinnon *et al.*, 1985). The rat's sciatic nerve (Figure 6) has an advantage in terms of surgical approach: it provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and insertion of grafts or guide tubes. For that reason it's still the most employed experimental model (van Neerven *et al.*, 2012).

Figure 6 – Surgical approach to the rat's sciatic nerve.

An appropriate animal model choice is fundamental for pre-clinical studies and must precede any clinical trial. Experimentally induced injuries including focal crush or freeze injury that cause axonal interruption but preserves the connective sheaths (axonotmesis), complete transection disrupting the whole nerve trunk (neurotmesis) and resection of a nerve segment inducing a gap of a certain length, are common procedures in these research area (Maurício *et al.*, 2011).

Adequate measures must be done to minimize pain and discomfort, human endpoints for animal suffering and distress must be taken in account since animal welfare is an important and serious issue. All procedures must be performed with the approval of the Local Country Veterinary Authorities in accordance with the European Communities Council Directive of November 1986 (86/609/EEC).

Surgeries are performed using an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). Under deep anaesthesia, the right sciatic nerve is exposed through a skin incision extending from the greater trochanter to the distal mid-half of the femur followed by a muscle splitting incision. For axonotmesis, after nerve mobilisation, a standardized crush injury is performed using a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria, figure 7A) exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3 mm lesion (Figure 7B) (Luís *et al.*, 2007; Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b).

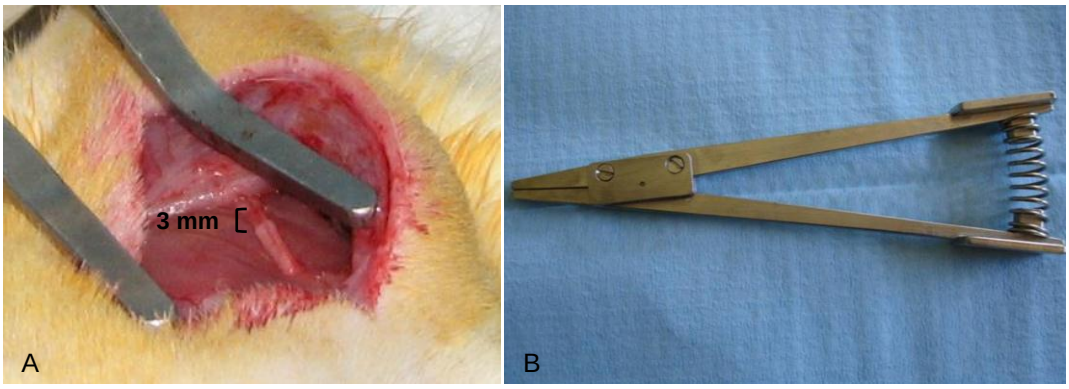

Figure 7 – (A) 3mm axonotmesis lesion (B) Non-serrated clamp (Luís, 2008) (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria).

For neurotmesis a transection injury is performed using straight microsurgical scissors. The nerve must be injured at a level as low as possible, generally immediately above the terminal nerve ramification**.** For neurotmesis with a nerve gap (the animal model of neurotmesis developed in our studies includes a gap of 10 mm), the proximal and distal nerve stumps are inserted 3 mm into the tube guide and held in place, maintaining a nerve gap of 10mm (Figure 8A), with two epineural sutures using 7/0 monofilament nylon. For neurotmesis without a gap, the nerve is reconstructed with an end-to-end suture (Figures 8B), with two epineural sutures using de 7/0 monofilament nylon. Finally the skin

and subcutaneous tissues are closed together with a simple-interrupted suture of a non-absorbable filament. An antibiotic should always be administered to prevent any infections. To prevent autotomy a deterrent substance must be applied to rats' right foot (Kerns *et al.*, 1991; Sporel-Ozakat *et al.*, 1991).

At this moment of state of art there are three surgical approaches for nerve reconstruction: (i) direct repair, (ii) nerve grafting and (iii) nerve transfer.

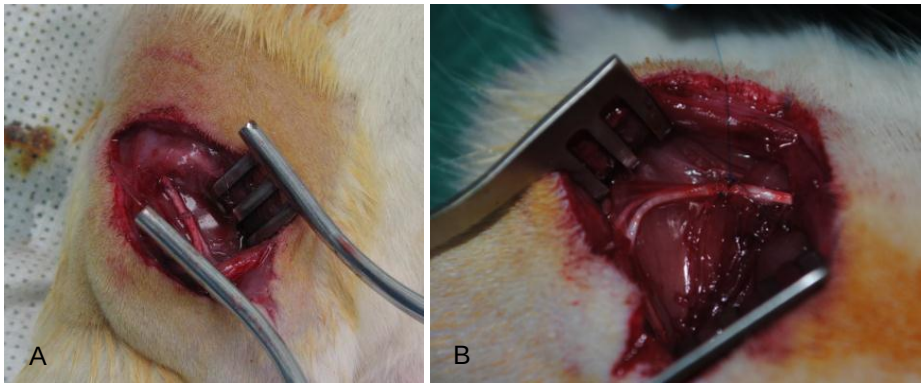

Figure 8 – Nerve reconstruction after neurotmesis lesion (A) nerve gap of 10mm (B) end-to-end suture

3.1.1. End-to-end suture

End-to end suture or direct repair is a suitable approach for reconnecting an injured nerve where no gap exists between the ends or when the suture can be performed without tension (Pfister *et al.*, 2011). At the best, if there is no scar tissue at the suture point, proximal axons will grow through the proliferating SCs into the distal segment promoting and directing regeneration. However, this strategy brings some difficulties in reproducing the original alignment of the nerve fascicles inducing some tension (Pfister *et al.*, 2011).

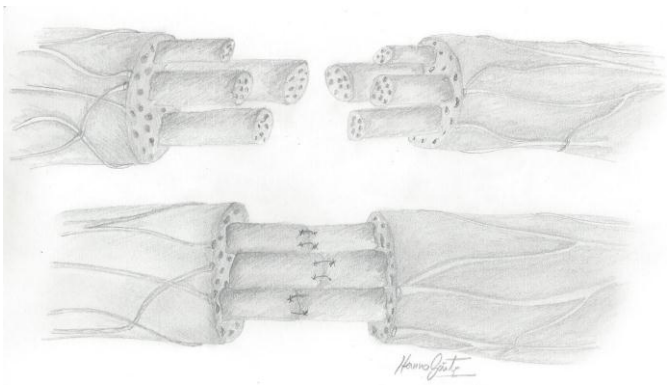

Figure 9 - Surgical reconstruction, end-to-end reconnection.

3.1.2. Autograft

To avoid excessive connective tissue formation and tension when re-joining nerve ends a bridging material is used. An autologous nerve grafting (nerve autograft) is the selected method and the gold standard in these injuries. Autografting is the best choice for severe peripheral gaps where direct coaptation is not possible without tension (Madduri *et al.*, 2010). Autografting carries several limitations; because it depends on the quality, quantity and appropriate size of donor's nerve. Nerve harvesting causes co-morbidity at donor site, loss of sensation, neuroma and scar formation, plus an insufficient revascularization of the graft may be the frequent reason for unsuccessful functional recovery (Madduri *et al.*, 2010; Keilhoff *et al.*, 2011).

3.1.3. Allograft

When autografts are not a possible choice, allografts and nerve conduits may be an option, as a good alternative for gaps between 3-10mm. In comparison with artificial nerve conduits these grafts have more biocompatibility, are less toxic and are more propitious to cell adhesion and growth. Nevertheless, allografts might undergo rejection so they require immunosuppressive therapies for at least two years, and carry many difficulties in isolation, so this strategy is typically reserved for cases with extensive or even irreparable nerve damages (Pfister *et al.*, 2011).

3.1.3.1. Non-autologous and acellular grafts

Non-autologous tissues have a great value for investigators as an attempt to outline the limitations of allografts. Allogeneic tissues from cadaveric donors and xenogeneic tissues from animal origin are available in large amounts and do not carry harvesting difficulties. Nevertheless rejection and consequent immunosuppression treatment remain as a concern and disease transmission may also be a problem to take under consideration (Schmidt *et al.*, 2003). To avoid immunorejection ablation of immunogenic components during the biomaterial processing is possible. Methods focus on removal or destruction of antigen cells maintaining the ECM components viable. Decellularization methods may be thermal, radiation or chemical processes (Schmidt *et al.*, 2003) which have been demonstrated not to be quite efficient. Thermal technique involves repeated cycles of freezing and heating, which has been efficient in cellular immunogenic destruction, leaving

however the ECM structure damaged and leading to inflammation. Radiation treatment has less damage on ECM, but the removal of cell debris is not complete. Chemical process that gives a good cellular clearance and maintain the ECM structure are the method with best results (Schmidt *et al.*, 2003). The use of acellular tissues in nerve repair may become a good alternative for the future.

4. Tissue engineering

Many *in vivo* studies with animal models have proven that nerves are capable to regenerate through various types of conduits. In the mainstream studies with rats, the nerve gaps ranged between 10-50mm in length. Concerning to the rat model, the sciatic nerve has been consensual for peripheral nerve regeneration studies. Rat and mouse, have been one of the most commonly used animal models for peripheral nerve regeneration studies due to their widespread availability as well as to the distribution of their nerve trunks which is similar to humans (Mackinnon *et al.*, 1985). There are a few studies where a 80mm gap was performed in other animal models like sheep (Strasberg *et al.*, 1996) and swine (Atchabahian *et al.*, 1998) but the axonal regeneration was very weak (Siemionow *et al.*, 2010). Other nerve trunks, especially in the rat forelimb, are getting more and more used for experimental research (Papalia *et al.*, 2006) but, the rat sciatic nerve is still the far most employed experimental model as it provides a nerve trunk with adequate length and space at the midthigh for surgical manipulation and introduction of grafts or guides (Rodriguez *et al.*, 2004; Maurício *et al.*, 2011). Sciatic nerve injuries are rare in humans, yet this experimental model provides a very realistic testing bench for injuries involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Mackinnon *et al.*, 1985).

4.1. Nerve conduits

The very first reported intent to use of a nerve conduit was in 1881 by Gluck who used a decalcified long bone (Ijpma *et al.*, 2008). In 1904 Foramitti defined nerve tubulation as "*guiding frame for the divided nerves, on which the newly emerging nerve substance can grow in the shortest way from the central to the peripheral stump without much resistance, and to protect the junction against embedding in callus and scar tissues, and to keep the nerve gap open for regeneration from the central into the peripheral stump"*. More than a century has passed and the concept is still the same (Ijpma *et al.*, 2008). The first nerve

regeneration study was performed by Mackinnon and Dellon using monkeys as a animal model. They used several length bio-absorbable conduits. It was proven that peripheral nerves could regenerate through a 3mm nerve gap with the help of an appropriate nerve conduit (Dellon *et al.*, 1988; Mackinnon *et al.*, 1990).

Artificial nerve conduits (NC) are presented as a promising alternative. Compared with autograft they have several advantages to be explored. Quantity is unlimited, size is not a restriction, as they can be produced in various sizes and shapes, and there is no donor-site morbidity. Many studies over the past years have studied different artificial NC, made of synthetic and natural material. However, the results still remain unsatisfactory. Yet, artificial nerve conduits in the past 30 years have progressed from a research tool to a real device to be used in clinical procedures as an alternative to autografts.

Physical and biological properties (Figure 10), such as permeability, flexibility, swelling, degradation, size and biocompatibility and biodegradability of nerve conduit material are very important for entubulation repair (de Ruiter *et al.*, 2009; Maurício *et al.*, 2011). Permeability is of extreme importance due to the necessity of oxygen and nutrient diffusion into the regeneration site (de Ruiter *et al.*, 2009). Porosity should be high enough to provide sufficient space for cell adhesion, extracellular matrix regeneration and the pore structure should allow spatial cell distribution throughout the scaffold to facilitate homogeneous tissue formation. A proper surface is also of vital importance because it must support cell adhesion, viability and tissue cell growth (Chen *et al.*, 2002). Furthermore, nerve conduits should exhibit a porous structure with interconnecting pores of suitable size to permit a rapid and sufficient vascularization. Penetration of blood vessel into the scaffolds and their efficient vascularization after implantation is a major precondition for long-term survival of the cells within the tissue construct (Laschke *et al.*, 2009). Flexibility is also an important property to facilitate de bridging of the gap, although this offers fragility to the conduit which may break or tear the suture (de Ruiter *et al.*, 2009).

The entubulation technique provides an environment for outgrowing axons and Schwann cells, which are indispensable elements for nerve functional recovery (Meek *et al.*, 2008). Tissue engineering needs to create a nerve conduit that can minimize fibrosis, permitting organized nerve regeneration, being able to guide the nerve fibres to their final organ and allowing nutritional supply (Keilhoff *et al.*, 2011).

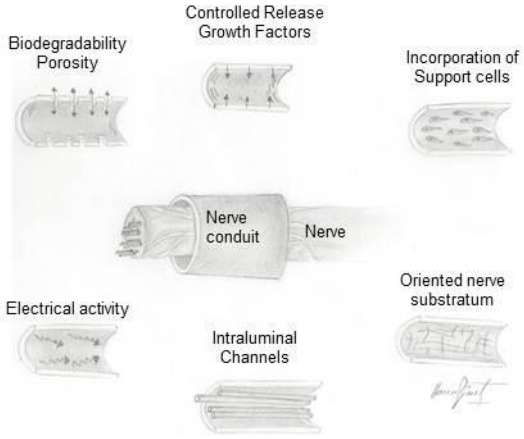

Figure 10 – Ideal physical and biological properties of nerve conduits.

Many research groups have focused their studies on synthetic nerve conduits. Compared with absence of surgical intervention, artificial nerve conduits significantly enhance regeneration (Bellamkonda, 2006). The first artificial nerve guide tested were non-reabsorbable silicone tubes, which required a second surgery to remove the synthetic material (Nectow *et al.*, 2012). Since then many different biomaterials have been tested and reabsorbable materials have the advantage to degrade in site and there is no need for second surgery to remove the material after regeneration of the tissues. The ideal nerve conduit should be fully degraded at the time of complete regeneration (Meek *et al.*, 2008). The degradation time is of extreme importance, if it's too quick before achievement of complete regeneration, there will be no support for the definitive connection of the two edges. On the other hand if it's too slow, it may cause compression at the site of regeneration inhibiting the whole process (den Dunnen *et al.*, 1995). A large number of trials using different materials as nerve conduits in animal models have been performed in the last years (Meek *et al.*, 2008). Nerve conduits should be easy to manage during surgery and be easily sterilized avoiding modification of physical properties.

4.2. Biomaterials

The ideal biomaterial for nerve conduit should be easy to: (i) design in different sizes; (ii) be sterilized and (iii) insert using microsurgical techniques. It must fulfil several biological and physicochemical requirements such as biocompatibility, biodegradability, permeability to ions and metabolites for the revascularization of the regenerated nerve, biomechanical and surface properties should enable and modulate the cellular system adhesion

(Maurício *et al.*, 2011). Biomaterials may be biological or synthetic; the second ones can be absorbable and non-absorbable (Schmidt *et al.*, 2003).

4.2.1. Natural

Natural derived biomaterial have the advantage that as biologically derived materials, they stimulate cell adhesion, migration, growth and proliferation, providing a special predisposition for regenerating injured nerve tissue. They are also biocompatible, don't induce foreign body reaction and the degradation debris are non-toxic. These interactions are mediated by specific receptor-binding sites with ECM molecules such as collagen, laminin, fibronectin and others. So, these components have become very important candidates for neural scaffolds, either as primordial elements of NC material or as a supplement to the NC, in the form of a hydrogel (Deumens *et al.*, 2010a). As mentioned before collagen is one of the most popular biological derived materials since it covers 30% of total body proteins, it is used either in soft and hard tissue engineering (Meek *et al.*, 2008; Deumens *et al.*, 2010a). Our research group has tested a type III equine collagen and hybrid chitosan in axonotmesis and neurotmesis injuries with very promising results. Collagen has demonstrated to be a good delivery vehicle for support cells (Luís *et al.*, 2008b; Amado *et al.*, 2010; Simões *et al.*, 2010). Chitosan has recently attracted particular attention due to its biocompatibility, biodegradability, low toxicity, low cost, wound-healing enhancement and antibacterial effects together with its potential use in nerve regeneration that have been demonstrated both *in vitro* and *in vivo* (Shirosaki *et al.*, 2005). In a previous study performed by our group, *in vivo* results showed that type III chitosan (hybrid chitosan) improved both morphological regeneration of nerve fibre and functional recovery. Chitosan type III is a hybrid of chitosan which was developed by the addition of γ-glycidoxypropyltrimethoxysilane (GPTMS). The addition of GPTMS improves the wettability of chitosan surfaces, and therefore it is expected to be more hydrophilic than the original chitosan. Chitosan type III was developed to be more porous, with a larger surface volume but still preserving mechanical strength and the ability to adapt to different shapes. Chitosan and chitosan-based materials have been proven to promote adhesion, survival, and neuritis outgrowth of neural cells (Amado *et al.*, 2008; Simões *et al.*, 2010).

4.2.2. Synthetic

4.2.2.1. Non-absorbable

In the beginning, the first nerve conduits to be used were non-absorbable silicone tubes, which required a second surgical intervention to remove the nerve guide (Nectow *et al.*, 2012). Other non-absorbable materials, such as porous stainless steel and elastomer hydrogel, have been reported, yet these materials carry some disadvantages. They induce foreign body reaction due to their characteristics, such as inflexibility and fibrous tissue formation therefore it is necessary to remove the conduit after recovery has been established (Nectow *et al.*, 2012).

4.2.2.2. Absorbable

Absorbable NCs are the ideal, once after the device has completed its mission; no foreign body material will be left behind. Among synthetic biodegradable materials, two types attracted particular attention to our research group: Poly(DL-lactide-ε-caprolactone) (PLC) (Varejão *et al.*, 2003a; Battiston *et al.*, 2005; Luís *et al.*, 2007a; Luís *et al.*, 2008a; Luís *et al.*, 2008b) and poly(lactic-co-glycolic acid) (PLGA) in a proportion 90:10 of the two polymers, poly(L-lactide): poly(glycolide) (PLA:PLG) (Luís *et al.*, 2007b; Luís *et al.*, 2008a). The PLC and the PLGA 90:10 exhibits several different physical-chemical properties, for instance PLC is stiffer than PLGA 90:10 due to structural reinforcement of the ester bonds and does not degrade so fast. The biodegradation rate of PLGA tubes is quicker, estimated to be around 3 months while the complete reabsorption of PLC is estimated to be of 16 months approximately (Luís *et al.*, 2007b; Luís *et al.*, 2008a; Geuna *et al.*, 2009). The biodegradation rate of PLGA is dependent on the proportion between the two polymers, PLA:PLG (Luís *et al.*, 2008a). The degradation products of PLC are less acidic, which may cause less damage to the surrounding tissue, and they are transparent facilitating the correct positioning of the nerve stumps, while PLGA tubes may compromise advantages in terms of microporosity, which may improve nerve repair. *In vitro* studies have shown that the PLGA, hybrid chitosan and PLC are biocompatible with nerve cells and may facilitate nerve cell attachment, differentiation and growth (Luís *et al.*, 2007b; Amado *et al.*, 2008; Luís *et al.*, 2008a; Geuna *et al.*, 2009). *In vivo* studies have demonstrated that PLGA and PLC improve significantly both motor and sensory functions. An important difference between both biomaterials was that the structure of the polymer was still well preserved, after 20 weeks, in nerves repaired by PLC while for PLGA guides the polymer was already substituted by a connective matrix rich in collagen fibres and

fibroblasts which looks like a normal epineurium. Nevertheless quantitative assessment of the total number of regenerated nerve fibres showed no statistical difference between nerves guided with PLC and PLGA (Luís *et al.*, 2007b; Luís *et al.*, 2008a).

Shin and colleagues reported in 2009 a study in which they compared 3 synthetic bioabsorbable conduits with a reversal autograft in sciatic nerve repair. One of the synthetic absorbable conduits was also a PLC tube which showed the best results equally with the autograft. The other two conduits demonstrated to be less effective (Shin *et al.*, 2009). In 2008 Meek and Coert published a review with an overlook through the FDA/CE approved absorbable conduits (Meek *et al*., 2008). The next table summarizes the characteristic of FDA/CE approved absorbable biomaterials for nerve conduits.

Table 4: FDA/CE approved absorbable nerve conduits

	NeuraGen	NeuroMatrix/NeuroFlex	Neurotube	Neurolac
Composition	Type I collagen	Type I collagen	Polyglycolic acid	Poly(DL-lactide-ε-caprolactone)
Company	Integra NeuroScience	Collagene Matrix Inc.	Synovis	Polyganics BV
Maximum length	3 cm	2.5 cm	4 cm	3cm
Diameter	1.5-7 mm	2-6 mm	2.3-8mm	1.5-10mm
Clinical Data	Yes	No	Yes	Yes
Average price	€1200	€600 (expected)	€340	€700-1800

*Adapted from (Meek *et al.*, 2008)

4.3. Supplements for Nerve Conduits

It is estimated that tissue engineering association of biomaterials to cellular systems, capable of differentiating into neuroglial-like cells, may improve peripheral nerve regeneration, in terms of motor, sensory and histomorphometric parameters.

In regard to supplements for conduits three main studies have been performed by several research groups around the world: (i) NC plus growth factors, (ii) NC cultured with SCs and (iii) NC cultured with different types of stem cells (Keilhoff *et al.*, 2011). It is known that neurotrophic factors are naturally released after a nerve cut, compromising a discriminating selection of neuron types (motor or sensory) and increase in axonal outgrowth and neuronal survival. For this reason since 1990 growth factors have been the focus of many researchers, as supplement to nerve conduits for improvement of nerve regeneration, (de Ruiter *et al.*, 2009). Nerve growth factor (NGF), brain-derived

neurotrophic factor (BDNF), ciliary neurotrophic factor (CNF) and insulin-like growth factor-1 (IGF-1) are some examples of natural growth factors that are produced by SCs and released during nerve regeneration (Siemionow *et al.*, 2010). Growth factors have the advantage to be soluble and easily incorporated into nerve conduits. *In vivo*, the method for growth factor delivery into injured site has a huge influence over the promotion and efficacy of nerve regeneration (Schmidt *et al.*, 2003). For this reason, it is important to develop strategies for growth factor delivery.

Polymer matrices, microspheres, and gene therapy are effective delivery methods. In what concerns to nerve regeneration, two delivery devices have been mainly used: polymer matrices and microspheres. For instance the nerve guidance channel is an example of a polymer matrix delivery system. They have been used as a model system to study peripheral nerve repair, by incorporating growth factors into the conduit wall, the nerve guidance channel itself becomes a delivery device. This system has a major problem; they only provide a finite reservoir of active biomolecular agents. Therefore, researchers are looking towards gene therapy techniques for a long-term production of active growth factors in situ (Schmidt *et al.*, 2003).

Floseal® is an example of a ready-to-use commercial haemostatic matrix with potential use in Tissue Engineering previously used with success by our research group for bone regeneration experiments (Lobato *et al.*, 2005). Floseal® is a haemostatic sealant composed of collagen-derived particles and topical bovine-derived thrombin and it has been proven to control bleeding in several medical applications, like vascular surgery, adenoidectomy, laparoscopy and partial nephrectomy. Floseal® is easily used and it can be extruded from a syringe and applied topically to the bleeding area. This haemostatic agent has the ability to acquire irregular shapes fitting the wounded site. When the Floseal® is in contact with blood the collagen particles are hydrated and swell, restricting the blood flow. The thrombin present converts the patient fibrinogen into a fibrin polymer, originating a clot around the lesion area of the nerve (Lobato *et al.*, 2005).

Our research group has tested the PLGA 90:10 and the hybrid chitosan type III tube-guides covered with N1E-115 differentiated *in vitro* during 48 hours in the presence of dimethylsul-foxide (DMSO), into neural-like cells. The N1E-115 cells derived from mouse neuroblastomas differentiate *in vitro* into neural-like cells and were used as a cellular model for stem cells (Maurício *et al.*, 2011). The purpose to cover the tube-guides with a cellular system differentiated *in vitro* into neural-like cells, was to locally produce neurotrophic factors. The role of neurotrophic factors in neural regeneration has been the focus of extensive research (Hu *et al.*, 1997; Amado *et al.*, 2008; Maurício *et al.*, 2011).

The influence of these factors in neural development, survival, outgrowth, and branching has studied from the molecular level to the macroscopic tissue responses. Neurotrophic factors promote a variety of neural responses, like the survival and outgrowth of the motor and sensory nerve fibres, and are implicated in peripheral nerve regeneration (Johnson *et al.*, 2008; Geuna *et al.*, 2009). N1E-115 cell line established from neuroblastoma, undergoes neuronal differentiation in response to dimethylsul-foxide (DMSO), adenosine 3';5'-cyclic monophosphate (cAMP), or serum withdrawal (Rodrigues *et al.*, 2005; Amado *et al.*, 2010) was a cellular system tested in axonotmesis and neurotmesis lesions to locally produce and deliver these neurotrophic factors. Results obtained by our research group using N1E-115 cells *in vitro* differentiated demonstrated that in what concerns to promoting axon regeneration there was a modest beneficial effect on functional recovery on axonotmesis injury (Luís *et al.*, 2008b) and for neurotmesis study evidences demonstrated negative effects on nerve fibre regeneration (Simões *et al.*, 2010; Maurício *et al.*, 2011). The N1E-115 cells present in nerve scaffolds probably compete for the nutrient and oxygen provided by local blood supply, these detail in association with the effect of space-occupying may have held up the positive effect of local neurotrophic factor release leading to a negative outcome on nerve regeneration, (Amado *et al.*, 2008; Simões *et al.*, 2010; Maurício *et al.*, 2011). Therefore, N1E-115 cells proved to be an unsuitable cellular system candidate for treatment of nerve injury after axonotmesis and neurotmesis, and its clinical application is limited in part due to its neoplastic origin (Maurício *et al.*, 2011).

The dynamic between SCs and axon growth is primordial for axon migration. SCs have a crucial role during Wallerian degeneration, producing ECM molecules, expressing cell adhesion molecules and secreting growth factors (Evans, 2001). Cultures of SCs have some intrinsic difficulties, such as culture time. In addition, and unless they are from autologous tissue, they also have the disadvantage, of being immunogenic therefore requiring immunosuppressive therapy. Even for autologous tissue they still have limitations like donor site morbidity and very little time for culturing autologous cells. Other cellular systems apart from SCs, have been proposed for supplement to NCs. Specially stem cell systems, since they overcome many of the issues responsible limited SCs harvesting.

5. Cellular Systems

5.1.

Cellular Systems and Biomaterials for Nerve Regeneration in Neurotmesis Injuries

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Cellular Systems and Biomaterials for Nerve Regeneration in Neurotmesis Injuries

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1. Introduction

A relevant number of peripheral nerve injuries can only be dealt through reconstructive surgical procedures. Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery. Particularly problematic is the fact that despite the good regenerative ability of peripheral nerves and successful surgical nerve repair functional recovery is most often disappointing in these patients (Lundborg, 2002). While direct nerve repair should be the procedure of choice whenever tension-free suturing is possible, in many cases there is a significant loss of nerve tissue and resulting nerve gap. In these cases a nerve graft might be necessary for adequate nerve repair (Lundborg, 2002). Nerve grafting, however have some disadvantages, the most prominent being donor site morbidity that may lead to a secondary sensory deficit and occasionally neuroma and pain. In addition, non-matching donor and recipient nerve diameters often occur, which might be at the basis of poor functional recovery (May, 1983). Entubulation offers advantages over autographs, including the potential to manipulate the regeneration environment within the tube-guide (Fields et al., 1989). Consequently, guidance of regenerating axons is not only achieved by a mechanical effect but also by a chemical effect (such as accumulation of neurotrophic factors) (Meek & Coert, 2002). Nerve guides can be made of biological or synthetic materials and, among the latter, both non-absorbable (e.g. silicon) and biodegradable tubes have been used (Schmidt & Leach, 2003). Biodegradable nerve guides must be preferred since no foreign body material will be left in

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the host after the device has fulfilled its task. Four types of nerve guides attracted particular attention of our research group: those made of PLGA (Luis et al., 2007b; Luis et al., 2008) and poly-ε-caprolactone (Luis et al., 2008; Varejao et al., 2003a), collagen (Amado et al., 2010) and chitosan (Amado et al., 2008). In particular, chitosan has recently attracted particular attention because of its biocompatibility, biodegradability, low toxicity, low cost, enhancement of wound-healing and antibacterial effects and its potential usefulness in nerve regeneration have been demonstrated both *in vitro* and *in vivo* (Amado et al., 2010; Shirosaki et al., 2005; Simoes et al., 2010). Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin (Senel & McClure, 2004). While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups, and the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. We have previously obtained by adding GPTMS, chitosan type III membranes (hybrid chitosan membranes) which have about 110 μm pores and about 90% of porosity, due to the employment of freeze-drying technique, and which were successful in improving sciatic nerve regeneration after axonotmesis (Amado et al., 2008). Wettability of the material surfaces is one of the key factors for protein adsorption, cell attachment and migration (Amado et al., 2008). The addition of GPTMS improves the wettability of chitosan surfaces, and therefore chitosan type III membranes are more hydrophilic than types of chitosan membranes (Shirosaki et al., 2005). In addition, chitosan type III was developed to be more porous, with a larger surface to volume ratio, and to preserve mechanical strength and ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported and it has been shown that they retain about two times as much biological fluid (Tateishi et al., 2002). The Neurolac® and the PLGA90:10 tube-guides guides show several different physical-chemical properties, namely Neurolac® is stiffer than PLGA due to structural reinforcement of the ester bonds and does not degrade so quickly. The biodegradation rate of PLGA tubes is estimated to be around 3 months while that complete resorption of Neurolac® is estimated to be of 16 months approximately (Geuna et al., 2009; Luis et al., 2007b; Luis et al., 2008). The degradation products of Neurolac® are less acidic, which may cause less damage to the surrounding tissue, and they are transparent so that the correct positioning of the nerve stumps may be confirmed after suturing. On the other hand, the biodegradable non woven structure of PLGA tubes may offer some advantages in terms of microporosity which may enhance nerve repair (Luis et al., 2007b; Luis et al., 2008). *In vitro* studies have shown that the four types of tube-guides, namely, the PLGA, the collagen, the hybrid chitosan and the Neurolac® are biocompatible to nerve cells and can facilitate nerve cell attachment, differentiation and growth (Amado et al., 2008; Luis et al., 2007b; Luis et al., 2008; Luis et al., 2008; Wang et al., 2008). Our research group tested the PLGA90:10, the hybrid chitosan type III and the collagen tube-guides covered with the neural cells *in vitro* differentiated for 48 hours in the presence of DMSO (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008; Simoes et al., 2010). The Neurolac® tubes were not tested with the cellular system, since the

cell adherence was more difficult to obtain and imply the previous covering with poli-l-lysine (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008; Simoes et al., 2010). It is expected that tissue engineering associating these biomaterials to cellular systems, capable of differentiating into neuron-like cells, may improve peripheral nerve regeneration, in terms of motor, sensory and histomorphometric parameters. The N1E-115 cells derived from mouse neuroblastomas (Amano et al., 1972) can *in vitro* differentiate into neural-cells (Amano et al., 1972; Rodrigues et al., 2005) and were used as a cellular model for stem cells. In addition, mesenchymal cells isolated from the Wharton's jelly of the umbilical cord, were used by our research group (data not published yet). Stem cells can be loosely classified into 3 broad categories based on their time of isolation during ontogenesis: embryonic, fetal and adult (Marcus & Woodbury, 2008). Recent years have witnessed an explosion in the number of adult stem cells populations isolated and characterized. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues. Recently, and currently controversial studies have challenged this dogma, suggesting that adult stem cells may be far more plastic than previously appreciated (Marcus & Woodbury, 2008). Extra-embryonic tissues as stem cell reservoirs offer many advantages over both embryonic and adult stem cell sources. Extra-embryonic tissues, collectively known as the afterbirth, are routinely discarded at parturition, so little ethical controversy attends the harvest of the resident stem cell populations. Most significantly, the comparatively large volume of extra-embryonic tissues and ease of physical manipulation theoretically increases the number of stem cells that can be isolated (Marcus & Woodbury, 2008). The umbilical cord contains two arteries and one vein protected by a proteoglycan rich connective tissue called Wharton's jelly. Within the abundant extracellular matrix of Wharton's jelly resides a recently described stem cell population called Umbilical Cord Matrix Stem Cells. It can be isolated around 400,000 cells per umbilical cord, which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow. *In vitro* the Wharton's jelly MSCs cells are capable of differentiation to multiple mesoderm cell types including skeletal muscle and neurons (Fu et al., 2006; Wang et al., 2004). Human MSCs (HMSCs) isolated from Wharton's jelly of the umbilical cord can be easily and ethically obtained and processed compared with embryonic or bone marrow stem cells. These cells may be a valuable source in the repair of the peripheral nerve system. HMSCs from Wharton's jelly of the umbilical cord possess stem cell properties (Yang et al., 2008) and it was previously demonstrated that HMSCs could be induced to differentiate into neuron-like cells (Fu et al., 2006). The transplanted cells were able to promote local blood vessel formation and expression of the neurotrophic factors, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Wang et al., 2004). Therefore, the purpose to cover the PLGA 90:10, hybrid chitosan, and collagen tube-guides used in reconstructed nerves after neurotmesis with a cellular system differentiated *in vitro* into neuronal cells, was to produce locally neurotrophic factors in a physiological concentration. The role of neurotrophic factors in neural regeneration has been the focus of extensive research (Amado et al., 2010; Hu et al., 1997). The influence of these factors in neural development, survival, outgrowth, and branching has been explored on various levels, from the molecular level to the macroscopic tissue responses. Neurotrophic factors promote a variety of neural responses, like the survival and outgrowth of the motor and sensory nerve fibers, and are implicated in spinal cord and peripheral nerve regeneration (Geuna et al., 2009; Johnson et al., 2008). However, *in vivo*, the efficacy of neurotrophic factors might vary greatly due to the method used for their delivering and it is thus

important to deliver them near the regenerating site, in the physiologic concentrations. In this view, association of a cellular system producing neurotrophic factors to biodegradable membranes may greatly improve the nerve regeneration in neurotmesis (Geuna et al., 2009). N1E-115 cell line established from a mouse neuroblastoma (Kerns et al., 1991), might be a useful cellular system to locally produce and deliver these neurotrophic factors (Geuna et al., 2001). *In vitro*, the N1E-115 cells undergo neuronal differentiation in response to dimethylsulfoxide (DMSO), adenosine 3′, 5′- cyclic monophosphate (cAMP), or serum withdrawal (Koka & Hadlock, 2001; Luis et al., 2007b; Luis et al., 2008; Luis et al., 2008). Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable (Koka & Hadlock, 2001; Luis et al., 2007b; Luis et al., 2008). The ideal interval period of 48 hours of differentiation was determined by measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$), when the N1E-115 cells presented already the morphological characteristics of neuronal cells but at a time, cell death due to increased $[Ca^{2+}]_i$ was not still occurring (Rodrigues et al., 2005).

2. Biomaterial for tube-guides fabrication

The tube-guides or scaffold of tissue engineered nerve grafts serve in neurotmesis injuries to direct axons sprouting from the proximal to the distal stump, to maintain adequate mechanical support for the regenerating nerve fibers. Yet, they provide a conduit channel for the diffusion of neurotrophic and neurotropic factors secreted in the regeneration local and provide a conduit wall for the exchange of nutrients and waste products with the surrounding media, limiting the infiltration of fibrous scar tissue that hinders axonal regeneration (Johnson & Soucacos, 2008;). The neural scaffolds should be easy to be fashioned in different lengths and diameters, to be sterilized and implanted using microsurgical techniques. The neural scaffold has to satisfy many biological and physicochemical requirements like biocompatibility, biodegradability, permeability to ions, metabolites and for the revascularization of the regenerated nerve, biomechanical properties and surface properties that modulate the cellular system adhesion (Huang & Huang, 2006). A wide variety of biomaterials has been attempted which are of either natural or synthetic origin.

2.1 Natural biomaterials

The natural biomaterials are known by stimulating cell adhesion, migration, growth and proliferation. The natural biomaterials have also a very good biocompatibility and less toxic effects. Our research group has tested the collagen and the chitosan.

Collagen, laminin and fibronectin play an important role in the development and growth of axons (Grimpe & Silver, 2002). So, these components have become very important candidates for neural scaffolds, these materials can also serve as delivery vehicles for support cells, growth factors and drugs. For example, silicone tubes filled with laminin, fibronectin, and collagen led to a better regeneration over a 10 mm rat sciatic nerve gap compared to empty silicone controls (Chen et al., 2007). Collagen filaments have also been used to guide regenerating axons across 20–30 mm defects in rats (Itoh et al., 2003; Yoshii & Oka, 2001). Further studies have shown that oriented fibers of collagen within gels, aligned using magnetic fields, provide an improved template for neurite extension compared to randomly oriented collagen fibers (Ceballos et al., 1999). Finally, rates of regeneration comparable to those using a nerve autograft have been achieved using collagen tubes

containing a porous collagen-glycosaminoglycan matrix (Archibald et al., 1995; Chamberlain et al., 2000). In our studies we have used equine collagen type III membrane (GentaFleece®; Baxter, Nuremberg, Germany) in axonotmesis and neurotmesis lesion of the rat sciatic nerve with very promising results (Amado et al., 2010; Luis et al., 2008).

Chitin is the second most abundant polysaccharide found in nature next to cellulose. It is a biopolymer of N-acetyl-D-glucosamine monomeric units and it has been used in a wide range of biomedical devices. Chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine and it has a very similar molecular structure with lamin, fibronectin and collagen. So, the chitosan has favorable biological properties for the nerve regeneration and it is easier to process than the chitin. Chitosan is quite fragile in its dry form, so, it has to undergo chemical cross-linking or to be used with other biomaterials before scaffold fabrication. The chitosan (high molecular weight, Aldrich®, USA) tested by our research group was dissolved in 0.25M acetic acid aqueous solution to a concentration of 2% (w/v). To obtain type III membranes, GPTMS (Aldrich®, USA) was also added to the chitosan solution and stirred at room temperature for 1h. The drying process for type III chitosan membrane was as follows: the solutions were frozen for 24h at -20°C and then transferred to the freeze-dryer, where they were left 12h to complete dryness. The chitosan type III membranes were soaked in 0.25N sodium hydroxide aqueous solution to neutralize remaining acetic acid, washed well with distilled water, and freeze dried (Amado et al., 2008). All membranes used in vivo testing, were sterilized with ethylene oxide gas, considered by some authors the most suitable method of sterilization for chitosan membranes (Marreco et al., 2004). Prior to their use *in vivo*, membranes were kept 1 week at room temperature to clear any ethylene oxide gas remnants (Amado et al., 2008; Simoes et al., 2010) (see Fig.1).

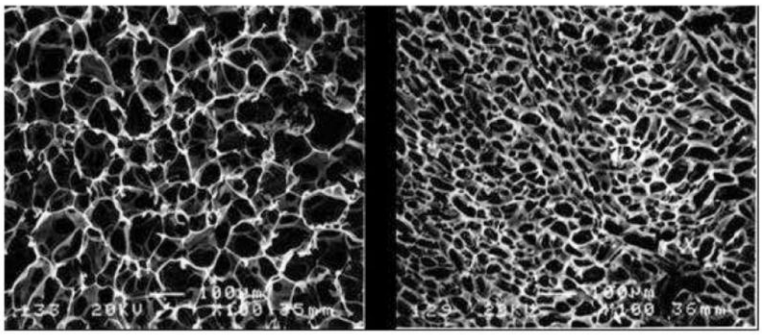

Fig. 1. SEM microstructure of chitosan membranes. Type II chitosan membrane (A). Type III chitosan membrane, showing a more porous microstructure, when compared to type II chitosan membrane (B) (Amado et al., 2008).

2.2 Synthetic biomaterials

The synthetic biopolymers constitute a group of promising biomaterials for the fabrication of neural tube-guides. However, the biocompatibility of some synthetic materials can be low since the difficulty of cell adhesion and survival can be a negative issue. Historically, non degradable synthetic materials were tested before the degradable ones. For instance, the silicone has been tested for peripheral nerve repair since 1960. Although it is not degradable in the body and it is impermeable to large molecules, the silicone tube-guide was an

important model system for studying nerve regeneration. It was also applied in several clinical trials to bridge short nerve gaps with some success (Gu et al., 2011). The use of non-degradable tube-guides implies a second surgery time, which has clear disadvantages for the patient. In order to overcome the disadvantages associated with non-degradable materials, research has been focused in biodegradable synthetic biomaterials that should degrade within a reasonable time span. The degradation products absorbed should not be toxic or induce a foreign body reaction. Moreover, the physiochemical and biological properties of biodegradable synthetic materials can be tailored to match different application requirements, like to entrap some molecules or serve as a support for a cellular system. Aliphatic polyesters represent a class of the common degradable synthetic polymers, among which poly(L-lactic acid) (PLLA) (Wang et al., 2009), polyglycolic acid (PGA) (Waitayawinyu et al., 2007), polycaprolactone (PCL) (Mligiliche et al., 2003) and their copolymers, including poly(lactic-ε-caprolactone (Neurolac®) (Den Dunnen et al., 1998; Luis et al., 2007b) and poly(L-lactic-co-glycolic acid (PLGA) (Bini et al., 2004; Luis et al., 2007b; Luis et al., 2008). Among synthetic biodegradable tubes, two types attracted particular attention: those made of PLGA and those made of Poly (DL-lactide-ε-caprolactone) copolyester (Neurolac®) (see Fig.2).

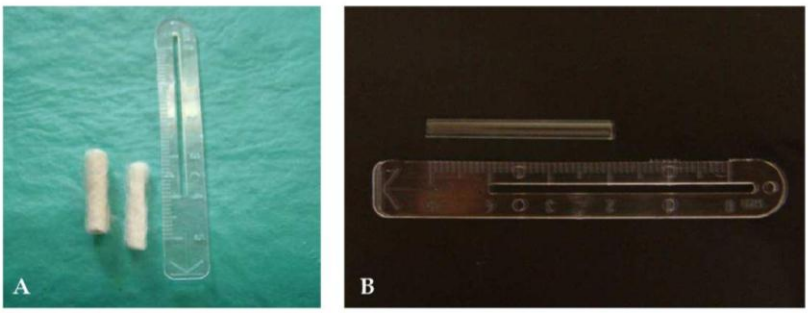

Fig. 2. PLGA 90:10 (A) and poly (DL-lactide-e-caprolactona) copolyester (Neurolac®) tube-guides (B) (Luis et al., 2007b).

These two types of nerve guides showed several different physical-chemical properties. The Poly (DL-lactide-ε-caprolactone) tube-guides (Neurolac®), 16 mm long, internal diameter of 2 mm and thickness wall of 1.5 mm, were purchased from Polyganics BV, Groningen, The Netherlands (Luis et al., 2007b; Luis et al., 2008). Poly (dl-lactide-co-glycolide) copolymers with ratio of 90PLA:10PLG were obtained from their cyclic dimers, dl-lactide and glycolide. Non-woven constructs were used to prepare tube-guides 16 mm long, internal diameter of 2.0 mm and thickness wall of 1.5mm, to be applied in a 10-mm sciatic nerve gap. These fully synthetic non-woven materials are extremely flexible, biologically safe and are able to sustain the compressive forces due to body movement after implantation. They have also some degree of porosity to allow for influx of low molecular nutrients required for nerve regeneration. The non-woven structure allowed the tube-guide to hold suture without difficulties, however greater care had to be taken in order to ensure its integrity. These tube-guides of PLGA are expected to degrade to lactic and glycolic acids through hydrolysis of the ester bonds (Luis et al., 2007b; Luis et al., 2008).

The polyvinyl alcohol hydrogel (PVA) tube-guides are now starting to be tested by our research groups. Previous studies showed the PVA potential to several biomedical

applications (Grant et al., 2006). The PVA gathers a set of exceptional properties, based in a nanofibrilar 3D structure, namely its high biocompatibility: it practically does not induce any foreign body or inflammatory reaction; it has high capacity to absorb water, mechanical resistance, elasticity, mobility and suturability (Bichara et al., 2010). The PVA are prepared in a tubular form, with 16 mm long, an internal diameter of 2 mm and thickness wall of 1.5 mm. These forms are prepared by a casting route to a mould that allows the final shape and thickness. Among the several methods described for the preparation of PVA material, the physical cross-linking of PVA by cyclic freezing and thawing is the one with better mechanical results (data not published by our research group). PVA from Aldrich (Mowiol 10-98: Mw 61,000, 98.0-98.8 mol% hydrolysis) is used. The tube-guides are produced using a solution of 20%(m/v) of PVA submitted to 3 repetitions of a freeze/thaw cycle, using 8 hours of freezing at –20.0°C and 4 hours of thawing at 22°C. However, several freeze/thaw cycling schemes must be tested to evaluate their effect on the final physical and mechanical properties, and choose the best one. The possible involvement of a final annealing step in the production process is being considered. This type of nerve guides can have unlimited availability in terms of diameters and lengths.

3. Cellular systems

The use of neural scaffolds alone to repair peripheral nerve defects have achieved variable success, but only small gap nerve repair (of 10 mm in rat sciatic nerve and of 30 mm in primate ulnar nerve) demonstrated evident functional and morphologic recovery (Amado et al., 2010; Gu et al., 2011; Hood et al., 2009; Luis et al., 2007b; Luis et al., 2008; Simoes et al., 2010). When the length of nerve gaps are increased, neural scaffolds alone only permit the bridging between the stumps, but for an effective regeneration, supporting cells or growth factors should be incorporated. The cellular systems implanted into the injury nerve may produce growth factors or ECM molecules, or may modulate the inflammatory process, to improve nerve regeneration (Amado et al., 2010; Gu et al., 2011; Luis et al., 2007b; Luis et al., 2008; Simoes et al., 2010). Schwann cells, mesenchymal stem cells, embryonic stem cells, marrow stromal cells are the most studied candidates of support cells among others. We focused our research in N1E-115 cell line *in vitro* differentiated (Amado et al., 2010; Luis et al., 2007b; Luis et al., 2008; Simoes et al., 2010) and in mesenchymal stem cells from Wharton's jelly of the umbilical cord (data not published yet).

To implant cultured cells (N1E-115 cells, MSCs, Schwann cells, and other cellular systems) into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected to the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). In alternative, implant can also be achieved by pre-adding the cells to the neural scaffold via injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve (Amado et al., 2010; Luis et al., 2008; Luis et al., 2008; Simoes et al., 2010).

3.1 Cell culture of N1E-115 cells – a cellular model for stem cells transplantation

The role of neurotrophic factors in neural regeneration has been the focus of extensive research in nerve regeneration (Schmidt & Leach, 2003). The influence of these factors in neural development, survival, outgrowth, and branching has been explored on various

levels, from molecular interactions to macroscopic tissue responses. One family of neurotrophic factors, the neurotrophins, has been heavily investigated in nerve regeneration studies, including the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Lundborg et al., 1994). Neurotrophic factors promote a variety of neural responses: survival and outgrowth of the motor and sensory nerve fibers, spinal cord and peripheral nerve regeneration (Schmidt & Leach, 2003). However, *in vivo* responses to neurotrophic factors can vary due to the method of their delivering. Therefore, the development and use of highly controlled delivery devices are required for the study of these extremely complex systems. For that reason, N1E-115 cell line established from neuroblastoma, that undergo neuronal differentiation in response to dimethylsul-foxide (DMSO), adenosine 3';5'-cyclic monophosphate (cAMP), or serum withdrawal (Amado et al., 2010; Rodrigues et al., 2005) was a cellular system tested in axonotmesis and neurotmesis lesions to locally produce and deliver these neurotrophic factors. Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable (Amado et al., 2010; Rodrigues et al., 2005). During differentiation, cyclin-dependent kinase (cdk) activities decline and phosphorylation of the retinoblastoma gene product (pRb) is lost, leading to the appearance of a pRb-containing E2F DNA-binding complex. The molecular mechanism by which pRb inhibits cell proliferation is becoming increasingly clear. pRb inhibits the activity of proteins that function as inducers of DNA synthesis (Kranenburg et al., 1995). Ca^{2+} serves as an important intracellular signal for cellular processes such as growth and differentiation. Free calcium levels within neural cells control many essential neural functions including neurotransmitter release, membrane conductance, nerve fibber excitability, coupling between neuronal cells, and axonal transport. Although regulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is important for normal cell functioning, its deregulation has been linked to cellular pathologies and cell death (Trump & Berezesky, 1995). Deregulation in $[Ca^{2+}]_i$ can be toxic to cells and is involved in the triggering of events leading to excitotoxic cell death in neurons, through the activation of calpain, phospholipases and endonucleases (Smith & Hall, 1988). Because of the importance of $[Ca^{2+}]_i$ in neuronal health and disease, a relatively simple cell model system, where $[Ca^{2+}]_i$ regulation can be studied fairly easily, is desirable. We have tested a non-expensive and easy method to culture neural-like cell line capable of producing locally, nerve growth factors and of growing inside PLGA90:10, chitosan and collagen tubular/membrane nerve guides (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008; Luis et al., 2008; Simoes et al., 2010), in order to use them to promote nerve regeneration across a peripheral nerve gap. To correlate the neuronal cells' ability to promote nerve regeneration across a gap, through their differentiation grade and survival capacity, the $[Ca^{2+}]_i$ of non-differentiated and DMSO differentiated N1E-115 cells was determined by the epifluorescence technique using the Fura-2-AM probe (Tsien, 1989). The measurement of $[Ca^{2+}]_i$ permitted to determine an ideal period of differentiation of 48 hours, when the N1E-115 cells presented already the morphological characteristics of neuronal cells and at the same time, the death process was not initiated by the $[Ca^{2+}]_i$ modifications. This cellular system was used in axonotmesis and neurotmesis injuries associated to chitosan, equine collagen type III and PLGA membranes or tube-guides in order to improve the functional and morphologic recovery of the nerve (Amado et al., 2010; Gu et al., 2011; Luis et al., 2007b; Luis et al., 2008; Simoes et al., 2010).

3.2 Wharton's jelly mesenchymal cells

After peripheral nerve injury, many neurons die. Because neurons cannot be easily expanded in vitro, there is a wide difficulty in applying primary cultured neurons to nerve tissue engineering. Embryonic stem cells (ESCs) have a great potential to proliferate unlimitedly and differentiate into neural cells using several differentiation protocols (Gu et al., 2011). The differentiation of ESCs can be modulated by growth factors and retinoic acid (Gu et al., 2011). Extensive research has been focused on the implantation of these stem cells in Central Nervous System (CNS) disorders, but in Peripheral Nerve System (PNS) injuries only a few studies were published. Cui and co-workers, in 2008, implanted ESC-derived neural progenitor cells into a 10-nm sciatic nerve gap, resulting in substantial axonal regrowth and nerve repair (Cui et al., 2008). The implanted cells survived for 3 months and could differentiate into myelinating cells. The nerve stumps presented almost normal diameter with longitudinally oriented, densely packed Schwann cell-like arrangement. The regenerated nerves also showed recovery of the functional activity, determined by electrophysiological records (Cui et al., 2008). Although these promising results, the ESCs have serious ethical issues, especially when obtained by somatic nuclear transfer technique or from embryos produced for assisted medical reproduction.

Recent years have witnessed a great expansion in the number of adult stem cell populations isolated and characterized. Adult stem cells have long been considered restricted, considering their multipotency. Many studies have challenged this dogma, suggesting that adult stem cells may be more plastic than previously appreciated (Beer et al., 2001). Extra-embryonic tissues as stem cell reservoirs offer many advantages over embryonic and adult stem cell sources, and are routinely discarded at parturition. Little ethical controversy attends the harvest of the resident stem cell populations. Human umbilical cord (UC) consists of three tissue components including the (1) amniotic membrane, (2) stroma (namely, Wharton's jelly), and (3) blood vessels (two arteries and one vein). The stroma is also further divided into three histological zones including the (1) subamniotic zone, (2) intervascular zone, and (3) perivascular zone (Can & Karahuseyinoglu, 2007). Wharton´s jelly (WJ) is a proteoglycan rich connective tissue of the UC. Within the abundant extracellular matrix of WJ resides a recently described stem cell population called UC Matrix Stem Cells. Can be isolated around 400,000 cells per UC, which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow. Isolated MSCs express CD29 and CD54, consistent with a mesenchymal cell type, and can be propagated in culture for more than 80 population doublings (Marcus & Woodbury, 2008). *In vitro* the WJ MSCs cells are capable of differentiation to multiple mesodermal cell types. This cells have been successfully differentiated into various cell types including adipocytes (Karahuseyinoglu et al., 2008), chondrocytes (Baksh et al., 2007; Wang et al., 2004), osteocytes (Baksh et al., 2007; Conconi et al., 2006; Wang et al., 2004), cardiomyocytes (Kadivar et al., 2006; Wang et al., 2004), skeletal myocytes (Conconi et al., 2006), hepatocytes (Kadivar et al., 2006), insulin-producing cells (Wu et al., 2009) as well as neuron-like cells (Fu et al., 2006; Weiss et al., 2006) (see Fig.3).

Generation of clinically important dopaminergic neurons has been also reported by other groups (Fu et al., 2006; Wang et al., 2004). MSCs isolated from WJ may be a valuable cellular system to improve peripheral nerve regeneration, since they possess stem cell properties and are able to differentiate *in vitro* into neuron-like cells (Fu et al., 2006; Yang et al., 2008). The transplanted cells are able to promote local blood vessel formation and to locally

produce neurotrophic factors like BDNF and GDNF (Wang et al., 2004). Transformed MSCs are still viable 4 months after transplantation without the need for immunological suppression, suggesting them as good source for Regenerative Medicine (Fu et al., 2006).

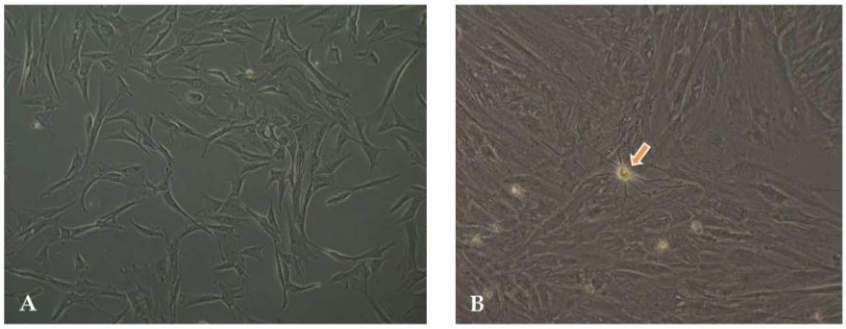

Fig. 3. Undifferentiated mesenchymal stem cells, from Wharton's jelly, exhibiting a star-like shape with a flat morphology (A) and neural-like cells, differentiated after 96 hours with neurogenic medium, formation of typical neural-like multi-branches (B) (100X magnification).

4. Microsurgical procedures

Rodents, particularly the rat and the mouse, have become the most frequently utilized animal models for the study of peripheral nerve regeneration because of the widespread availability of these animals as well as the distribution of their nerve trunks which is similar to humans (Mackinnon et al., 1985; Rodriguez & Navarro, 2004). Although other nerve trunks, especially in the rat forelimb, are getting more and more used for experimental research (Papalia et al., 2006), the rat sciatic nerve is still the far more employed experimental model as it provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and/or introduction of grafts or guides (Rodriguez & Navarro, 2004). Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Mackinnon et al., 1985). Common types of experimentally induced injuries include focal crush or freeze injury that causes axonal interruption but preserves the connective sheaths (axonotmesis), complete transection disrupting the whole nerve trunk (neurotmesis) and resection of a nerve segment inducing a gap of certain length. For these reasons our experimental work, concerning the *in vivo* testing of neurotmesis injury regeneration, was based on the use of Sasco Sprague-Dawley rat sciatic nerve. Usually, the surgeries are performed under an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). Under deep anaesthesia (ketamine 9 mg/100 g; xylazine 1.25 mg/100 g, atropine 0.025 mg/100 body weight, intramuscular), the right sciatic nerve is exposed through a skin incision extending from the greater trochanter to the distal mid-half followed by a muscle splitting incision. After nerve mobilisation, a transection injury is performed (neurotmesis) using straight microsurgical scissors, at a level as low as possible, in general, immediately above the terminal nerve ramification. The proximal and distal nerve stumps are inserted 3 mm into the tube-guide (not covered or covered with cells) and

held in place, maintaining a nerve gap of 10mm, with two epineurial sutures using 7/0 monofilament nylon. For neurotmesis without gap, the nerve is reconstructed with an end-to-end suture, with two epineural sutures using de 7/0 monofilament nylon. Finally the skin and subcutaneous tissues are closed with a simple-interrupted suture of a non-absorbable filament (Synthofil®, Ethicon). An antibiotic (enrofloxacin, Alsir® 2.5 %, 5 mg / kg b.w., subcutaneously) is always administered to prevent any infections. To prevent autotomy a deterrent substance must be applied to rats' right foot (Kerns et al., 1991; Sporel-Ozakat et al., 1991). All procedures must be performed accordance with the European Communities Council Directive of November 1986 (86/609/EEC).

5. Functional evaluation

Our studies of sciatic nerve regeneration after neurotmesis include a post-surgery follow-up period of 20 weeks based on the assumption that, by the end of this time, functional and morphological recovery are complete (Amado et al., 2010; Luis et al., 2007b; Luis et al., 2008; Simoes et al., 2010). Although both morphological and functional data have been used to assess neural regeneration after induced neurotmesis and axonotmesis injuries, the correlation between these two types of assessment is usually poor (Dellon & Mackinnon, 1989; Shen & Zhu, 1995). Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labelling (Mackinnon et al., 1985; Mackinnon SE, 1988) do not necessarily predict the reestablishment of motor and sensory functions (de Medinaceli et al., 1982; Shen & Zhu, 1995). Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery (Shen & Zhu, 1995). In this sense, research on peripheral nerve injury needs to combine both functional and morphological assessment. The use of biomechanical techniques and rat's gait kinematic evaluation is a progress in documenting functional recovery (Varejao et al., 2003b). Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles (Varejao et al., 2003b; Varejao et al., 2002).

5.1 Evaluation of motor performance (EPT) and nociceptive function (WRL)

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively. The animals are tested pre-operatively (week-0), at weeks 1, 2, and every two weeks thereafter until week-20. The EPT was originally proposed by Thalhammer and collaborators, (Thalhammer et al., 1995) as a part of the neurological recovery evaluation in the rat after sciatic nerve injury. For this test, the entire body of the rat, excepting the hind-limbs, is wrapped in a surgical towel. Supporting the animal by the thorax and lowering the affected hind-limb towards the platform of a digital balance, elicits the EPT. As the animal is lowered to the platform, it extends the hind-limb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance is recorded. The same procedure is applied to the contra-lateral, unaffected limb. Each EPT test must be repeated 3 times and the average result is considered. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into a equation (Equation 1) to derive the percentage of functional deficit (Koka & Hadlock, 2001).

$$\text{Percentage motor deficit} = [(\text{NEPT} - \text{EEPT}) / \text{NEPT}]*100$$

The EPT data, originally measured in grams of weight borne by each hindlimb, is expressed as percentage deficit from total bearing weight as determined by the weight borne by the unoperated limb. Potential pitfalls of the EPT do exist. It takes a certain training period for the tester to become comfortable handling the animals, and this comfort level is critical for the animal to behave in a non frightened way. There is also a level of recognition of when the animal is bearing its maximum weight, which is critical since the tester is supporting the body of the animal at all times. Once this recognition takes place, through training by an experienced tester, the examination becomes highly reproducible (Koka & Hadlock, 2001).
To assess the nociceptive withdrawal reflex (WRL), the hotplate test was modified as described by Masters and collaborators (1993). The rat is wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate at 56°C and with the other on a room temperature plate. WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. Normal rats withdraw their paws from the hotplate within 4.3 s or less (Hu et al., 1997). The affected limbs are tested 3 times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies are averaged to obtain a final result (Campbell, 2001). If there is no paw withdrawal after 12 s, the heat stimulus is removed to prevent tissue damage, and the animal is assigned with the maximal WRL of 12 s (Varejao et al., 2003a).

5.2 Sciatic functional index (SFI) and static sciatic index (SSI)

For SFI, animals are tested in a confined walkway measuring 42-cm-long and 8.2-cm-wide, with a dark shelter at the end. A white paper is placed on the floor of the rat walking corridor. The hind paws of the rats are pressed down onto a finger paint-soaked sponge, and they are then allowed to walk down the corridor leaving its hind footprints on the paper. Often, several walks are required to obtain clear print marks of both feet. Prior to any surgical procedure, all rats are trained to walk in the corridor, and a baseline walking track is recorded. Subsequently, walking tracks are recorded pre-operatively (week-0), at weeks 1, 2, and every two weeks thereafter until week-20. Several measurements are taken from the footprints: (I) distance from the heel to the third toe, the print length (PL); (II) distance from the first to the fifth toe, the toe spread (TS); and (III) distance from the second to the fourth toe, the intermediary toe spread (ITS). The static footprints are obtained at least during four occasional rest periods. In the static evaluation (SSI) only the parameters TS and ITS, are measured. For both dynamic (SFI) and static assessment (SSI), all measurements are taken from the experimental and normal sides. Four steps should be analyzed per rat. Prints for measurements should be chosen at the time of walking based on clarity and completeness at a point when the rat was walking briskly. The mean distances of three measurements are used to calculate the following factors (dynamic and static):

$$\text{Toe spread factor (TSF)} = (\text{ETS} - \text{NTS})/\text{NTS}$$

$$\text{Intermediate toe spread factor (ITSF)} = (\text{EITS} - \text{NITS})/\text{NITS}$$

$$\text{Print length factor (PLF)} = (\text{EPL} - \text{NPL})/\text{NPL}$$

Where the capital letters E and N indicate injured and non-injured side, respectively.
The SFI was calculated as described by Bain et al. (1989) (Bain et al., 1989) according to the following equation:

$$SFI = -38.3\ (EPL - NPL)/NPL + 109.5(ETS-NTS)/NTS + 13.3(EIT-NIT)/NIT - 8.8 = (-38.3 \times PLF) + (109.5 \times TSF) + (13.3 \times ITSF) - 8.8$$

The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation (Bervar, 2000; Meek et al., 2004)

$$SSI = [(108.44 \times TSF) + (31.85 \times ITSF)] - 5.49$$

For both SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given. In each walking track three footprints must be analyzed by a single observer, and the average of the measurements are used in SFI calculations.

5.3 Kinematic analysis

Locomotion is also of higher functional relevance since it involves integrated function of both the motor and sensory systems and their respective components, such as skeletal muscles, sensory endings, efferent and afferent nerve fibers and integrative centers within the central nervous system. Muscles innervated by sciatic nerve branches include both dorsiflexors and plantarflexors and, although in previous studies we focused our kinematic analysis only in the stance phase (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008), we now prefer to include analysis of the ankle joint motion also during the swing phase in order to provide additional information (Joao et al., 2010).

In our previous studies we analyzed ankle kinematics in the sagittal plane during the stance phase of walking either after sciatic nerve transection and repair (Amado et al., 2010; Luis et al., 2008) or after sciatic nerve crush (Amado et al., 2008). The two-dimensional (2D) ankle motion analysis was conducted during voluntary level walking along a corridor with darkened cages at both ends to attract the animals. The lateral walls of the corridor were made of Perspex and a high speed video camera (JVC GR-DVL9800, Nex Jersey, USA) was placed orthogonally to corridor length in order to record ankle motion during walking. Sagittal records of the rat walking were obtained at a frame rate of 100 frames per second and images were semi-automatically digitized using marks at reference points of the rat hindlimb and foot (see Fig. 4B).

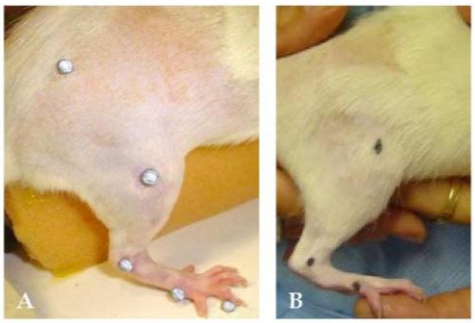

Fig. 4. Reflective markers with 2mm diameter were attached to the right hindlimb at bony prominences (from bottom to top): (1) tip of fourth finger, (2) head of fifth metatarsal, (3) lateral malleolus, (4) lateral knee joint, (5) trochanter major (A). Tattoo marks at bony prominences of the right hindlimb: (1) head of fifth metatarsal, (2) lateral malleolus, (3) lateral knee joint (B).

The trajectories of the segments leg and hindfoot were obtained through this procedure and ankle joint angle was derived by using dot product computation. Ankle kinematics parameters proposed by Varejão et al. (2003) were employed in order to assess functional recovery after sciatic nerve transection and repair and to compare treatments using our tested biomaterials and cellular system. Our ankle joint kinematic analysis generally showed profound walking dysfunction in the weeks after sciatic neurotmesis. Changes affected the whole stance phase and were best characterized by an inability of the animals to perform the normal ankle push off action during the second half of this walking phase (see Fig 5 and Fig.6). Profound ankle kinematic changes during the stance phase of walking were also evident when taking into account angular velocity data. Uninjured animals show a clear ankle angular velocity peak both at the initiation and end of the stance phase. After sciatic transection these two peaks are not observed and the stance phase is characterized by a rather steady increase in positive ankle velocity that corresponds to a progressive augment in ankle dorsiflexion. This abnormal pattern of ankle motion during the stance phase of walking was interpreted as caused by denervation and paralysis of the ankle plantarflexors that were unable to actively extend the ankle against the load of the body weight. In sciatic nerve transected and repaired animals, the recovery of the normal ankle motion pattern of walking is slow and largely incomplete. After 20 weeks of recovery, abnormal ankle motion is still noticed after sciatic nerve neurotmesis (Amado et al., 2010; Luis et al., 2008). In our recent study (Amado et al., 2010), investigating the role of a collagen membrane with and without differentiated N1E-115 cells enwrapped around the transected and end-to-end repaired sciatic nerve, the shape of ankle joint motion during the stance phase showed little improvements until three months after injury (see Fig 5 and Fig.6).

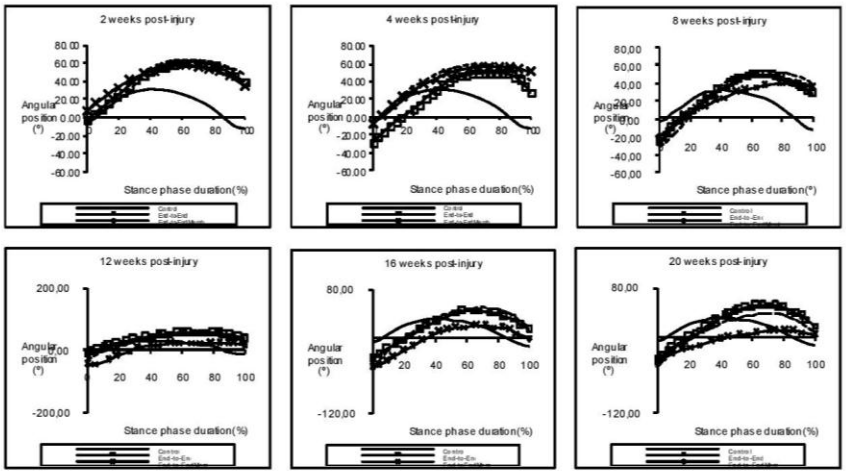

Fig. 5. Kinematics plots in the sagittal plane for the angular position (°) of the ankle as it moves through the stance phase, during the healing period of 20 weeks. The mean of each group is plotted.

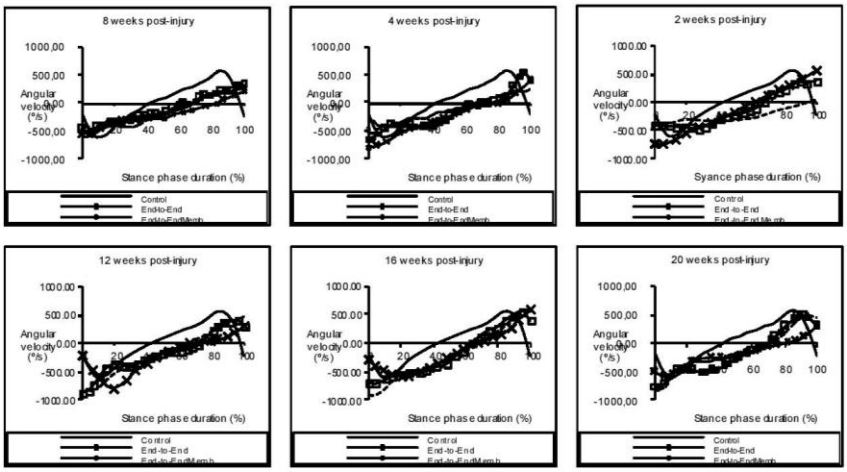

Fig. 6. Kinematics plots in the sagittal plane for the angular velocity (°/s) of the ankle as it moves through the stance phase, during the healing period of 20 weeks. The mean of each group is plotted.

A progressive improvement in ankle kinematics occurred from this time point up to the end of the 20 weeks recovery time. Such improvement consisted mainly in a slight recovery of ankle plantarflexion at the end of stance phase and lesser peak dorsiflexion at midstance. These changes in ankle kinematics are suggestive of muscle reinnervation and increased force generation ability by the ankle pantarflexor muscle group. In this study, ankle kinematics data were in general agreement with the results of the EPT and WRL tests. The latter tests also demonstrated progressive recovery of motor and sensory function along the follow up time that in the case of the WRL test was accelerated beyond the 12 weeks time point. As for ankle kinematics, the EPT and WRL results also demonstrated incomplete functional recovery in these animals, with slightly better recovery in the end-to-end repair group compared to other two groups. However, ankle kinematics must be envisaged merely as an indirect measure of muscle function. It should be noticed that walking requires fine coordination of limbs motion and definitely quadruped animals have many movement strategies to compensate for deficit in one of the hindlimbs. Through plasticity of integrative structures, animals may develop adaptive patterns that persist even if significant reinnervation takes place. Also, no direct relation exists between more simple tests of muscle strength or of sensory function and a complex action such as walking (Varejao et al., 2003b). Indeed, ankle kinematics data poses a fundamental question. Does recovery of ankle motion during walking signals successful muscle reinnervation and regain of muscle function or else is a product of compensatory changes at whole hindlimb level? To answer this question satisfactorily, walking analysis has to be improved by extending the analysis to hip and knee joints and should be combined with direct measures of muscle activity (Gramsbergen et al., 2000a) and to measures of the force applied to the ground by the walking animal (Howard et al., 2000). To better assess hindlimb joint kinematics during walking, we recently analyzed hip, knee and ankle joint kinematics during recovery of less severe sciatic nerve crush injury, using a more sophisticated motion capture system to track the motion of reflective markers attached to the rat hindlimb (unpublished observations) (see Fig. 4A).

After this kind of injury, functional recovery is often deemed as complete even on the basis of ankle kinematic analysis with only minor walking changes observed after 12 weeks of recovery. However, after 12 weeks of recovery from complete sciatic nerve crush, changes in hip and knee kinematics during walking were present, when compared to sham-operated control animals. Importantly, such altered hip and knee kinematics were in contrast with total reestablishment of the normal pattern of ankle motion in sciatic-crushed animals.

Individual joint kinematics either in control or nerve-injured animals is characterized by high variability, with notable differences between different animals and even from step to step (Chang et al., 2009). Such high level of variability, which seems to be an intrinsic property of normal quadruped walking, seriously affects the precision of joint kinematic measures of functional recovery after nerve injury. Reducing this variability is a challenge for efficient use of walking analysis to assess functional recovery. Attempts to overcome this limitation include constraining walking velocity by using treadmill walking instead of self-paced locomotion (Pereira et al., 2006). This, of course, is likely to reduce step-by-step variability in joint kinematics but has the disadvantage of requiring expensive equipment and limits the possibility of combining kinematic analysis with other data, such as ground reaction forces. Other possibilities look at a global, limb-level movement analyses as an alternative to individual joints kinematics (Chang et al., 2009; Sabatier et al., 2011). Also, systematic changes in the biomechanical and movement control constraints of the locomotor task, such as using up- and down-slope walking might also increase the accuracy of walking analysis within the context of peripheral nerve research (Sabatier et al., 2011).

In summary, walking analysis is a promising method to assess functional recovery after hindlimb nerve injury. However, in order to provide accurate measures of functional recovery, walking analysis after hindlimb peripheral nerve injury will have to evolve from simply analyzing ankle kinematics to reach a full biomechanical description of hindlimb motion including analysis of hip, knee and ankle joints. Further refinements of walking analysis in the field of peripheral nerve research using the rat model will probably include the combined use of joint kinematics, ground reaction forces and electromyographical data of muscle activity.

6. Morphological analysis

It has been recently pointed out that morphological analysis is the far most common method for the study of peripheral nerve regeneration (Raimondo et al., 2009). Actually, the investigation of nerve morphology can give us important information on various aspects of the regeneration processes which relates with nerve function (Geuna et al., 2009).

Although different types of fixatives can be used for peripheral nerve histology, we fix nerve samples in a solution of 2.5% purified glutaraldehyde (Histo-line Laboratories s.r.l., Milano, Italy) and 0.5% saccarose (Merck, Darmstadt, Germany) in 0.1M Sörensen phosphate buffer, pH 7.4, for 6-8h. Nerves are then washed and stored in 0.1M Sörensen phosphate buffer added with 1.5% saccarose at 4-6°C prior to embedding. Sörensen phosphate buffer is made with 56g di-potassium hydrogen phosphate 3-hydrate (K2HPO4-3H2O) (Fluka, Buchs, Switzerland) and 10.6 g sodium di-hydrogen phosphate 1-hydrate (NaH2PO4-H2O) (Merck, Darmstadt, Germany) in 1 litre of doubly-distilled water. Just before the embedding, nerves are washed for few minutes in the storage solution and then immersed for 2 h in 2% osmium tetroxide (Sigma, St.Louis, MO) in the same buffer solution. The specimens are then carefully dehydrated in passages in ethanol and embedded in

Glauerts' mixture of resins which is made of equal parts of Araldite M and the Araldite Härter, HY 964 (Merck, Darmstad, Germany). At the resin mixture, 2% of accelerator 964, DY 064 is added (Merck, Darmstad, Germany). Finally, the plasticizer (0.5% of dibutylphthalate) is added to the resin.

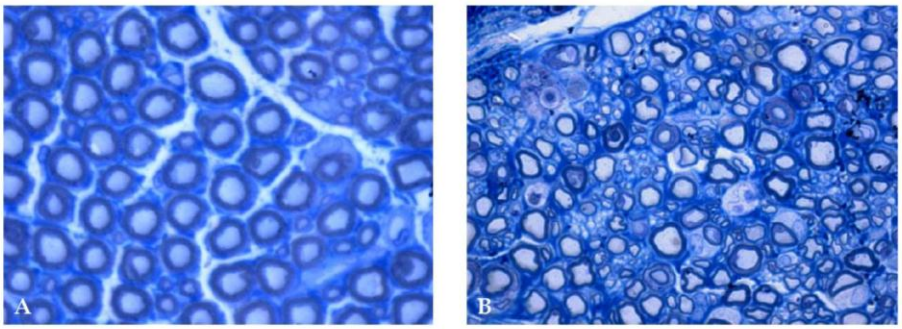

Fig. 7. Representative high resolution photomicrographs of nerve fibers from normal (A) and regenerated (B) rat sciatic nerves after entubulation with collagen nerve guide. Original magnification = 1,000x.

In our laboratory, histomorphometry (stereology) is carried out on toludine-blue-stained semi-thin sections (2.5 micron-thick) of nerve samples using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). We adopt a final magnification of 6,600x in order to enable accurate identification of myelinated nerve fibers (Figure 9). A 2D-disector method, (Raimondo et al., 2009) is finally used for estimating the total number of myelinated fibers (N), the mean diameter of fiber (D) and axon (d) as well as mean [(D-d)/2] and g-ratio (D/d) (see Fig.7).

7. Synopsis of results and discussion

Peripheral nerve regeneration can be studied in a number of different experimental models based on the use of nerves from both forelimb and hindlimb (Hu et al., 1997). The experimental animal model of choice for many researchers remains the rat sciatic nerve. It provides an inexpensive source of mammalian nervous tissue of identical genetic stock that it is easy to work with and well studied (Geuna et al., 2009) and shows a similar capacity for regeneration in rats and sub-humans primates (Johnson et al., 2008). The rat sciatic nerve is a widely used model for the evaluation of motor as well as sensory nerve function at the same time. One of the most addressed issues in experimental nerve repair research is represented by entubulation (Chen et al., 2006). While early studies were more directed towards biological material (Kerns et al., 1991) recent studies have focused more on synthetic materials that are biodegradable. The majority of natural biomaterials used in clinical applications such as hyaluronic acid, collagen, and gelatin are derived from animal sources. In spite of thorough purification methods these materials bear the inherent risk of transfer of viral and prionic diseases and may cause immunological body reactions (Koka & Hadlock, 2001; Luis et al., 2007b). Synthetic biomaterials are not associated with these risks (Koka & Hadlock, 2001; Luis et al., 2007b).

Our group has assessed two types of nerve guides made of a different type of biodegradable synthetic biomaterial, PLGA and caprolactone (Neurolac®). Our data showed that both types of nerve guides were a mean to help in the growth of axons and didn't deleteriously interfere with the nerve regeneration process. While the information on the effectiveness of Neurolac® tubes for allowing nerve regeneration was already provided experimentally (; Geuna et al., 2009; Luis et al., 2008) and with patients (Bertleff et al., 2005), the PLGA tubes with the polymers proportion of 90 PLA:10 PLG used in that study have never been tested *in vivo* before (Luis et al., 2007b). Results of the comparative functional assessment showed that the differences between the two experimental groups were not significant in terms of motor and nociceptive recovery. The EPT proposed by Thalhammer and coworkers (Geuna, 2005) to evaluate the motor function in rats is used routinely by veterinarian neurologists to evaluate the nervous system of small domestic animals. This reflex is initiated by a stretching of the spindles in the interosseous muscles and stimulation of sensory receptors of the foot (Lundborg, 2002). A steady recover of motor deficit occurred throughout the 20-week post-surgery period in the group of animals where PLGA and Neurolac® tube-guides were used. As expected, the motor recovery in animals of the end-to-end group occurred significantly faster and to a larger extent when compared to the entubulation groups. Similarly, nociception recovered to a significantly larger extent in the end-to-end group compared to both entubulation groups which, on the contrary, did not differ significantly between them (Luis et al., 2007b). Interestingly, when the nerve is transected and the regenerating axons must bridge a gap, sensory neurons exhibit a faster regenerative pattern than motor neurons (Lundborg, 2002). Morphological analysis showed a different pattern of biodegradation of the two tubes. In fact, while in the nerves repaired by Neurolac® the structure of the polymer was still well preserved at week-20, in the nerves repaired by PLGA guides, the polymer was largely substituted by a connective matrix rich in collagen fibbers and fibroblasts resembling a normal epineurium. These results may suggest that PLGA can be a better biomaterial for fashioning nerve guides since nerves regenerated inside it were more similar to a normal nerve than those regenerated inside Neurolac® guides, although the results of the motor and sensory functional assessment did not disclose significant differences between the two tube-guides and thus both should be considered a good substrate for preparing tubular nerve guides. We have also used the rat sciatic nerve model for investigating the effects of chitosan type III membranes after neurotmesis followed by surgical repair either by direct suture, or autograft or tubulization. Morphological results showed that nerve regeneration occurred when the chitosan type III tube-guide was used and that, at time of withdrawal, Wallerian degeneration was almost completed and substituted by re-growing axons and the accompanying Schwann cells. The results obtained with chitosan type III tube-guides were significantly better, in terms of functional and morphologic recovery, when compared with PLGA90:10 where the regeneration pattern was worse (Simoes et al., 2010). The synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III compared to common chitosan, as well as the presence of silica ions, may be responsible for the good results in promoting post-traumatic nerve regeneration (Amado et al., 2008) suggesting that this material may not just work as a simple mechanical scaffold but instead may work as an inducer of nerve regeneration (Amado et al., 2008). The neuroregenerative property of chitosan type III might be explained

by a direct stimulation of Schwann cell proliferation, axon elongation and myelination (Shirosaki et al., 2005). Yet, the expression of established myelin genes such as PMP22, PO and MBP (Pietak et al., 2007) may be influenced by the presence of silica ions which exert an effect on several glycoprotein expression (Pietak et al., 2007). Taken together, results of this study support the view that hybrid chitosan type III membranes can be a valuable tool for fashioning nerve guides aimed to bridge nerve defects. We also investigated the effects on nerve regeneration of the employment PLGA90:10 nerve guide tubes covered with N1E-115 cells *in vitro* differentiated in the presence of DMSO for 48 hours (Geuna et al., 2001). Results showed that both motor and sensory functions improved significantly similarly to what occurs in PLGA tubes alone (Luis et al., 2008). Nerve recovery of around 60% was achieved by week-20, in both groups, reconstructed with PLGA 90:10 tube-guides with or without the cellular system. The pattern of degradation and the degradation products of PLGA90:10 more acidic than the collagen ones do not seem to influence negatively the degree of nerve regeneration during the healing period. On the other hand, the biodegradable non-woven structure of PLGA tubes may offer some advantages in terms of microporosity which may enhance nerve repair. It is not surprising that recovery was significantly better in the group where the gap was reconstructed using the autologous graft since this is still considered the gold standard for peripheral nerve regeneration (Matsuyama et al., 2000). The rationale for the use of the N1E-115 cells was to take advantages of the properties of these cells as a neural-like cellular source of neurotrophic factors (Geuna et al., 2001).

Although tube guides are a suitable choice for peripheral nerve reconstruction, it is known that nerve regeneration and functional recovery are less satisfactory than when nerve repair is done by using an end-to-end neurorraphy or when an autologous nerve graft is applied (Geuna et al., 2009). For example, the risk of neuroma formation in neurotmesis injury is considerable, which is clearly avoided with tube-guide technique. In this sense, enhancing the rate of axonal growth might prevent the occurrence of side effects and might turn nerve regeneration faster, thus improving functional recovery. Neurotrophic factors play an important role in nerve regeneration after injury or disease and it is conceivable that if neurotrophic factors are applied in the close vicinity of the injured nerve their healing potency is optimized. In spite of these premises and contrary to our initial hypothesis, the N1E-115 cells did not facilitate either nerve regeneration or functional recovery and, as far as morphometrical parameters are concerned, results showed that the presence of this cellular system reduced the number and size of the regenerated fibers. These results suggest that this type of nerve guides can partially impair nerve regeneration, at least from a morphological point of view (Luis et al., 2008). The impaired axonal regeneration seems to be the result of N1E-115 cells surrounding and invading the regenerating nerve, since numerous of these cells where seen colonizing the nerve and might have deprived regenerated nerve fibers blood supply (Luis et al., 2008). The use of N1E-115 cells did not promote nerve healing and their use might even derange the nerve regenerating process. Whereas the effects on nerve regeneration were negative, an interesting result of this study was the demonstration that the cell delivery system that we have used was effecting in enabling long-term colonization of the regenerated nerve by transplanted neural cells. Whether the negative effects of using N1E-115 cells as a cellular aid to peripheral neural tissue regeneration extends to other types of cells is not known at present and further studies are warranted to assess the role of other cellular systems, e.g. mesenchymal stem cells, as a foreseeable therapeutic strategy in peripheral nerve regeneration. Our

experimental results with this cellular system are also important in the perspective of stem cell transplantation employment for improving posttraumatic nerve regeneration with patients (Luis et al., 2008). Undoubtedly, great enthusiasm has raised among researchers and in the general public about cell-based therapies in regenerative medicine (Geuna et al., 2009; Thalhammer et al., 1995). There is a widespread opinion that this type of therapy is also very safe in comparison to other pharmacological or surgical therapeutic approaches. By contrast, recent studies showed that cell-based therapy might be ineffective for improving nerve regeneration (Varejao et al., 2003b) or even have negative effects by hindering the nerve regeneration process after tubulisation repair. Whereas the choice of the cell type to be used for transplantation is certainly very important for the therapeutic success, our present results suggest that the paradigm that donor tissues guide transplanted stem cells to differentiate in the direction that is useful for the regeneration process it is not always true and the possibility that transplanted stem cells choose another differentiation line potentially in contrast with the regenerative process should be always taken in consideration.

8. Acknowledgements

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5.2.

Mesenchymal Stem Cells from Extra-Embryonic Tissues for Tissue Engineering

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Chapter 18

Mesenchymal Stem Cells from Extra-Embryonic Tissues for Tissue Engineering – Regeneration of the Peripheral Nerve

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Additional information is available at the end of the chapter

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1. Introduction

Recent advances in Regenerative Biology and Regenerative Medicine are impressive and in the last years the scientific community has witnessed the emergence of many new concepts and discoveries. Until a few years ago, biological tissues were regarded as unable of extensive regeneration, but nowadays organs and tissues like the brain, spinal cord or cardiac muscles appear as capable to be reconstructed, based on "stem cells" [1].

Stem cell research has sparked an international effort due to the variety of possible uses in clinical procedures to treat diseases and improve health and life expectancy. Stem cell research has crossed a century journey and has evolved greatly even in its own definition. In 1967, Lajtha defined that adult stem cells could only be found in regenerative organs, such as blood, intestine, cartilage, bone and skin. Nowadays, these cells are considered to exist even in tissues with no commitment to regeneration such as the central nervous system [1, 2].

2. Stem cells

Stem cells are undifferentiated cells, with endless self-renewal sustained proliferation *in vitro* and multilineage differentiation capacity [3]. This *in vitro* multilineage differentiation capacity has targeted these cells with extreme importance for use in tissue and cell-based therapies.

The first stem cell appearance is in the early zygotic cells, which are totipotent and give rise to the blastocyst. They are capable to differentiate into all cell and tissue types. With differentiation, cells become less capable of self-renewal and differentiation in other cell type becomes more limited [1]. Stem cells can be loosely classified into 3 broad categories based on their growth behavior and isolation time during ontogenesis: embryonic, fetal and adult.

Embryonic stem cells (ESCs) were first observed in a pre-implantation embryo by Bongso and colleagues in 1994 [4]. Since then, many cell lines and a multiplicity of tissues have been successfully derived from ESCs and tested in several animal disease models [5-7]. Nevertheless, post-transplantation immune-rejection has been a major problem. Many studies are being conducted to avoid this major issue. This could be resolved by personalizing tissues through somatic nuclear transfer (NT) or induced pluripotent stem cells (iPSC) techniques [8], but the teratoma development in animals is still a concern and a serious problem [9]. In order to overcome the limitations placed by ESCs and iPSCs, a variety of adult stem cell populations have been recently isolated and characterized for their potential clinical use. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues [9]. *In vivo,* adult stem cells exist in a quiescent state, located in almost all tissues, until mediators activate them to restore and repair injured tissues. These cells are surrounded by mature cells that have reached the end line in terms of differentiation and proliferation [10]. Stem cell research focuses on the development of cell and tissue differentiation, so as characterization techniques, for tissue and cell identification with marker patterns. Such protocols are essential for regenerative therapies [11].

2.1. Mesenchymal stem cells

The development of cell-based therapies for cartilage [12] and skin [13] reconstruction marks the beginning of a new age in tissue regeneration. Mesenchymal stem cells (MSCs) have become one of the most interesting targets for tissue regeneration due to their high plasticity, proliferative and differentiation capacity together with their attractive immunosuppressive properties. MSCs present low immunogenicity and high immunosuppressive properties due to a decreased or even absence of Human Leucocyte Antigen (HLA) class II expression [14]. Research in this field has brought exciting promises in many disorders and therefore in tissue regeneration. Currently the differentiation potential of MSCs in multilineage end-stage cells is already proven, and their potential for treatment of cardiovascular [15], neurological [16], musculoskeletal [17, 18], and cutaneous [19] diseases is now well established. Fibroblast colony-forming units or marrow stromal cells, currently named MSCs,

were first isolated in 1968 from rat bone marrow [20]. These cells were clonogenic, formed colonies when cultured, and were able to differentiate *in vitro* into bone, cartilage, adipose tissue, tendon, muscle and fibrous tissue. Since then many other tissues have been used to isolate these cells. MSCs can be obtained from many different tissues, including bone marrow, adipose tissue, skeletal muscle, umbilical cord matrix and blood, placental tissue, amniotic fluid, synovial membranes, dental pulp, fetal blood, liver, and lung [21]. The concept of MSCs is based on their ability to differentiate into a variety of mesodermal tissues and was first proposed by Caplan in 1991 [22] and further validated by additional research in 1999 [23]. Due to the many different methods and approaches used for MSCs culture, the Mesenchymal and Tissue Stem Cell Committee, of the International Society for Cellular Therapy (ISCT), recommended several standards do define MSCs [24]. Therefore, MSCs are defined as presenting: i) plastic adherent ability; ii) absence of definitive hematopoietic lineage markers, such as CD45, CD34, CD14, CD11b, CD79α, CD19 and class-II Major Histocompatibility Complex (MHC) molecules, specially HLA-DR; and expression of nonspecific markers CD105, CD90 and CD73 iii) ability to differentiate into mesodermal lineage cells, osteocytes, chondrocytes and adipocytes. Along with mesodermal differentiation, it has been demonstrated the capacity of MSCs to differentiate into ectodermal cell lines, as neurons [25, 26], keratocytes [27] and keratinocytes [28], so as endodermal cell line, like hepatocytes [29, 30] and pancreatic β-cells [31]. Moreover, they also possess anti-inflammatory and immunomodulation properties and trophic effects [32, 33]. Increasing evidence now demonstrates that the therapeutic effects of MSCs do not lay only on the ability to repair damage tissue, but also on the capacity of modulating surrounding environment, by secretion of multiple factors and activation of endogenous progenitor cells [34, 35]. Compared with ESCs and other tissue specific stem cells, MSCs are more advantageous. Moreover some studies have demonstrated that MSCs have a higher chromosomal stability and lower tendency to form tumors and teratomas, compared to other stem cells [36, 37].

Although they present similar biological characteristics, it cannot be ignored the existing of some disparities, as differences in, expansion potential under same culture conditions and age-related functional properties [38]. Compared to ESCs, MSCs isolated from the umbilical cord matrix (Wharton's jelly) have many advantages, such as shorter population doubling time, easy culture in plastic flasks, good tolerance towards the immune system, so that transplantation into non-immunesuppressed animals does not induce acute rejection, anticancer properties, [9] and most important absence of tumorigenic activity. As well as ESCs, these cells are originated from the inner cell mass of the blastocyst but with a major difference: they do not raise ethical controversies, since they are collected from tissues usually discarded at birth [39].

2.1.1. MSCs sources and validation of transport and processing protocols

Bone marrow, adipose tissue, umbilical cord blood and umbilical cord matrix have been considered the main sources of MSCs for tissue engineering purposes. Among these sources, bone marrow represents the main source of MSCs for cell therapy. However, the prolifera-

tive capacity [40-43], differentiation potential and clonal expandability [44] of MSCs derived from bone marrow decrease significantly with age, gender and seeding density, and the number of cells per marrow aspirate is usually quite low [3, 45]. It is still a mystery if MSCs ageing is due to factors intrinsic or extrinsic to the cells. Many possible reasons have been described in an attempt to explain MSCs ageing. Possible extrinsic factors include: reduced synthesis of proteoglycans and glycosaminoglycans reducing proliferation and viability [46], and production of glycosylated end products, inducing apoptosis and reactive oxygen species [47]. Intrinsic factors causing MSCs ageing might include: cell senescence-associated β-galactosidase and higher expression of p53 and pathway genes p21 and BAX, resulting in blunted proliferation potential [43]. Regarding seeding density, many authors suggest that lower seeding densities induce faster proliferation rates [48, 49]. This has been explained by contact inhibition in higher seeding densities [49], and higher nutrient availability per cell in lower seeding densities [49]. Use of bone marrow MSCs has disadvantages; donors are submitted to invasive harvest of bone marrow. This raises the need to find alternative sources of MSCs for autologous and allogenic use. Candidate tissue sources should provide MSCs displaying high proliferative and differentiation potency [50].

Extra-embryonic tissues are a good alternative to adult donor. This tissues, such as, amnion, microvillus, Wharton's jelly and umbilical cord perivascular cells, are routinely discarded at child-birth, so little ethical and religious controversy attends the harvesting of the resident stem cell populations. The comparatively large volume of extra-embryonic tissues increases the chance of isolating suitable amounts of stem cells, despite the complex and expensive procedures needed for their isolation. Some protocols use enzymatic digestion while others use enzyme-free tissue explant methods that require longer culture time [51]. There are also MSCs in cord blood (CB), but many studies report low frequency of these cells and unsuccessful isolation. However, Zhang and colleagues were able to isolate MSCs from CB with a 90% successful rate when CB volume was $\geq$ 90ml and a transport time until storage was $\leq$ 2 hours [51].

In recent years, MSCs derived from umbilical cord matrix Wharton's jelly, have attracted much interest. Wharton's jelly is a mature mucous tissue and the main component of the umbilical cord, connecting the umbilical vessels to the amniotic epithelium. Umbilical cord derives from extra-embryonic or embryonic mesoderm; at birth it weighs about 40g and measures approximately 30-65cm in length and 1.5cm in width [52]. Anyway, individual differences are observed within newborn babies. Fong and colleagues characterized Wharton's Jelly stem cells and found the presence of both embryonic and MSCs, targeting this source as unique and of valuable use for clinical applications. MSCs from the Wharton's jelly can be cultured with little or even no major loss trough at least 50 passages [53].

CB and more recently, umbilical cord tissue (UCT) have been stored cryopreserved in private and public cord blood and tissue banks worldwide in order to obtain hematopoietic and MSCs and, although guidelines exist (Netcord – Foundation for the Accreditation of Cellular Therapy), standardized procedures for CB and UCT transport from the hospital / clinic to the laboratory, storage, processing, cryopreservation and thawing are still awaited.

These may be critical in order to obtain higher viable stem cells number after thawing and limit microbiological contamination.

Our research group focused in determining whether UCT storage and transport from the hospital / clinical to the laboratory at room temperature (RT) or refrigerated (4-6°C) and immersed in several sterile saline solutions affects the UCT integrity in order to be cryopreserved. The umbilical cord contains two arteries and one vein, which are surrounded by mucoid connective tissue, and this is called the Wharton's jelly. The cord is covered by an epithelium derived from the enveloping amnion. The interlaced collagen fibers and small, woven bundles are arranged to form a continuous soft skeleton that encases the umbilical vessels. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid, which forms a hydrated gel around the fibroblasts and collagen fibrils and maintains the tissue architecture of the umbilical cord by protecting it from pressure [54].

One centimeter-long fragments of umbilical cords (N = 12) were collected from healthy donors after written informed consent and following validated procedures according to the clinical and technical guidelines of the Private Bank Biosckin, Molecular and Cell Therapies, SA (authorized for processing and cryopreserving CB and UCT units by the Portuguese Minister of Health, ASST – Autoridade para os Serviços de Sangue e de Transplantação). The 1 cm fragments were immersed for 168 hours in 4 different sterile saline solutions at RT (22-24°C) and refrigerated (4-6°C): NaCl 0.9% (Labesfal, Portugal), AOSEPT®-PLUS (Ciba Vision, Portugal), Dulbecco's Phosphate-Buffered Saline without calcium, magnesium and phenol red (DPBS, Gibco, Invitrogen, Portugal) and Hank's Balanced Salt Solution (HBSS, Gibco, Invitrogen, Portugal). The preservative-free, aqueous AOSEPT® PLUS solution contains hydrogen peroxide 3%, phosphonic acid (stabiliser), sodium chloride, phosphate (buffer system), and poloxamer (surfactant), and is usually used to transport and wash contact lenses. After 168 hours, the fragments were collected in 4% of paraformaldehyde and processed for light microscopy. The samples were fixed in 4% paraformaldehyde for 4 hours and then washed and conserved in phosphate buffer saline (PBS) until embedding. The specimens were dehydrated and embedded in paraffin and cut at 10 μm perpendicular to the main umbilical cord axis. For light microscope analysis, sections were stained with haematoxylin and eosin (HE) and observed with a Leica DM400 microscope equipped with a Leica DFC320 digital camera. The UCT integrity was evaluated through the following parameters:

i. detachment of vessels and retraction of vascular structures;

ii. loss of detail and integrity of the endothelium;

iii. connective tissue degradation;

iv. autolysis of fat (impossible to assess, due to histological technique); and

v. loss of detail and integrity of the mesothelium.

It was concluded that the best transport solutions were HBSS or DPBS at a temperature of 4-6°C since those maintained the histological structure of UC evaluated through those 5 parameters previously referred (Figure 1 and Figure 2).

Figure 1. Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6°C. Samples were stained with haematoxylin and eosin (HE). Magnification: 10X.

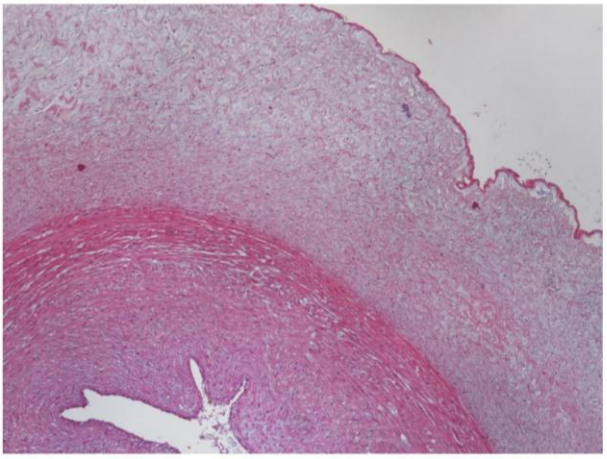

Figure 2. Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6°C. Samples were stained with haematoxylin and eosin (HE). The UCT integrity was quality evaluated through the following parameters: i) detachment of vessels and retraction of vascular structures; ii) loss of detail and integrity of the endothelium; iii) connective tissue degradation; iv) autolysis of fat (impossible to assess, due to histological technique); and v) loss of detail and integrity of the mesothelium. Magnification: 40X.

As a matter of fact, the UC immersed for 168 hours in DPBS and HBSS at refrigerated temperature presented integrity of the histological structure comparable to a UC collected and processed for histological analysis immediately after birth (Figure 3). With DPBS, a slight retraction of the vessels was noted, which is advantageous since the vessels are stripped and discarded before cryopreservation of the UCT. It was concluded that the transport of the UC

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from the hospital / clinic to the cryopreservation laboratory should be performed with the UC immersed in DPBS or HBSS at refrigerated temperatures.

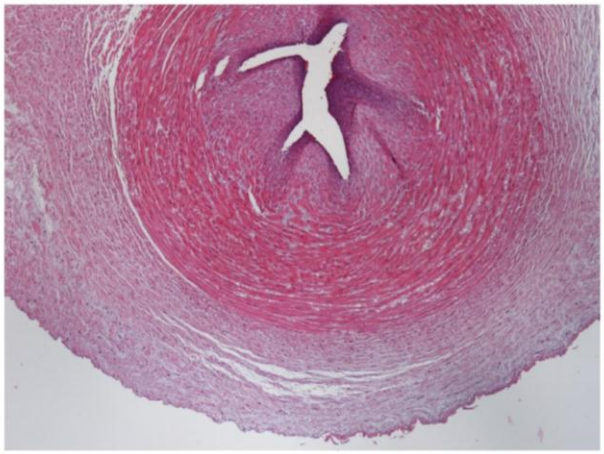

Figure 3. Cross section of an umbilical cord collected and processed for histological analysis immediately after birth under optimal conditions according to Netcord guidelines. Stained with haematoxylin and eosin (HE). Magnification: 40X.

The isolation and culture of MSCs from the Wharton's jelly was performed by our research group in order to obtain undifferentiated MSCs and *in vitro* differentiated into neural-like cells to be tested in axonotmesis and neurotmesis lesions of the rat sciatic nerve. The isolation has been performed by enzyme-free tissue explant and enzymatic isolation. Despite our standard approaches, we are aware that there are still significant variations that exist between laboratory protocols, which must be taken into account when comparing results using other methodologies. There is a wide range of individual differences among donor tissues also and our protocols usually use 15 - 20 cm of UC. While most UC samples will provide a reasonable number of MSCs using the provided protocols, some samples may result in sub-optimal cell isolation and expansion. The reasons behind this phenomenon still remain to be clarified, but as we have previously mentioned, the temperature and the time of transport from the hospital / clinic to the cryopreservation laboratory is crucial.

Irrespective of the specific protocol, the washing procedure of the umbilical cord fragments is crucial in order to avoid microbiological contamination of the cultures. After obtaining the written informed consent from the parents, fresh human umbilical cords are obtained after birth and collected in HBSS or DPBS at 4-6°C, as it was previously described. After washing the umbilical cord unit 4 times in rising DPBS, disinfection is performed in 75% ethanol for 30 seconds. Finally, and before the dissection step, umbilical cord unit is washed in DPBS. The vessels are usually stripped with UC unit still immersed in DPBS. Once washing step in MSCs isolation and culture is essential to achieve good UCT units for cryopreservation and future clinical use, washing protocol was validated. DPBS from the first washing step (used immediately after collection for transportation of the unit to the laboratory – *washing step 1 solution*) and

DPBS used in washing step after disinfection in 75% ethanol (*washing step 6 solution*) from 14 umbilical cord units (N = 14) collected from healthy donors and transported from the hospital/clinic at 4-6°C in less than 96 hours were tested for microbiological contamination using BacT/ALERT® (bioMérieux). Each unit was tested for aerobic and anaerobic microorganisms and fungi using 10 ml of the *washing step 1 solution* and *washing step 6 solution* which were aseptically introduced into the BacT/ALERT® testing flasks. All procedures were performed in a laminar flow tissue culture hood under sterile conditions. All the units that presented microbial contamination in DPBS obtained from the first washing step (*washing step 1 solution*) presented no contamination in the analysis performed to DPBS from the last washing step immediately performed before MSCs isolation or UCT cryopreservation (*washing step 6 solution*). The following microorganisms were identified in the DPBS solution from the first washing step: *Staphylococcus lugdunensis* (N = 2); *Staphylococcus epidermidis* (N = 1); *Staphylococcus coagulase* (N = 2); *Escherichia coli* (N = 4); *Enterococcus faecalis* (N = 1); and *Streptococcus sanguinis* (N = 1). The DPBS solution from the first washing step (*washing step 1 solution*) from 3 units was negative for microbial contamination (N = 3). These results permitted us to conclude that the washing protocol was 100% efficient in what concerns microbiological elimination (including aerobic and anaerobic bacteria, yeast and fungi).

Once the transport and washing protocols were validated, it was important to isolate and expand *in vitro* the MSCs from the UCT units for pre-clinical trials.

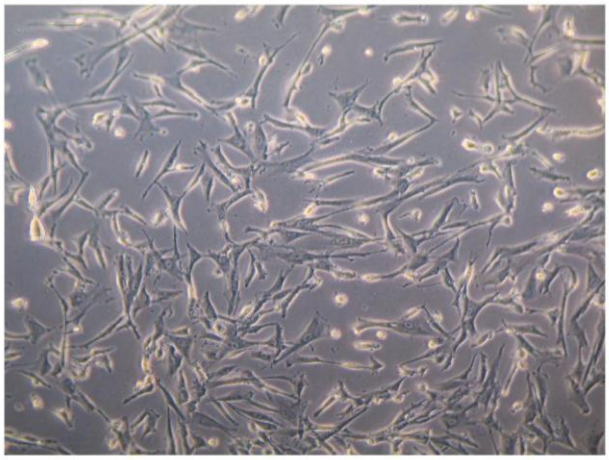

Figure 4. MSCs isolated from Wharton's jelly using the "enzymatic protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.

In the "enzymatic procedure" we use collagenase type I (Sigma-Aldrich). With the written informed consent from the parents, fresh human umbilical cords were obtained after birth and stored in HBSS (Gibco, Invitrogen, Portugal) for 1–48 hours before tissue processing to obtain MSCs. After removal of blood vessels, the mesenchymal tissue is scraped off from the Wharton's jelly with a scalpel and centrifuged at 250 g for 5 minutes at room temperature and the pellet is washed with serum-free Dulbecco's modified Eagle's medium (DMEM,

Gibco, Invitrogen, Portugal). Next, the cells are centrifuged at 250 g for 5 minutes at room temperature and then treated with collagenase (2 mg/ml) for 16 hours at 37°C,washed, and treated with 2.5% trypsin-EDTA solution (Sigma-Aldrich) for 30 minutes at 37°C with agitation. Finally, the cells are washed and cultured in DMEM (Gibco, Invitrogen, Portugal) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/l), 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) in 5% CO_2 in a 37°C incubator (Nuaire). Around 2×10^5 cells are plated into each T75 flask in 10 ml culture medium. Cells are allowed to attach and grow for 3 days. To remove the non-adherent cells or fragments, the flasks are gently washed using pre-warmed DPBS after which 10 ml of pre-warmed culture medium is added. The culture medium is changed every third day (or twice per week). Confluence (80-90%) is normally reached at day 12–16, and the cells are removed with pre-warmed trypsin-EDTA solution (4 ml per flask), for 10 min at 37°C. The cells are plated onto poly-l-lysine coated glass coverslips (in 6- or 24-well tissue culture plates) or on biomaterials used in the nerve reconstruction. Normally, 5000 cells/cm^2 are plated on the coverslips or on the membranes (Figure 4).

In our "enzyme-free tissue explant protocol" for isolation of MSCs, enzymatic digestion is not employed. The mesenchymal tissue (Wharton's jelly) is diced into cubes of about 0.5 cm^3 and the remaining vessels are removed by dissection. Using a sterile scalp, the cubes are diced in 1-2 mm fragments and transferred to a Petri dish pre-coated with poly-l-lysine (Sigma) with Mesenchymal Stem Cell Medium (PromoCell, C-28010) supplemented with 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) and cultured in 5% CO_2 in a 37°C incubator (Nuaire). Some tissue fragments will allow cell migration from the explants in 3-4 days incubation. Confluence is normally obtained 15-21 days after.

The laboratory's processing and cryopreservation protocols of the UCT units following the technical procedures of Biosckin, Molecular and Cell Therapies S.A. (BSK.LCV.PT.7) were validated for the ability of isolating and expanding *in vitro* MSCs after cryopreserved UCT thawing. The protocols of processing and cryopreservation of the UCT are protected by a Confidentiality Agreement between Biosckin, Molecular and Cell Therapies S.A. and all the involved researchers. Briefly, the UCT collected from healthy donors (N = 60), and according to Netcord guidelines and following the Portuguese law 12/2009 (*Diário da República, lei 12/2009 de 26 de Março de 2009*) is diced into cubes of about 0.5 cm^3 and the remaining vessels are removed by dissection. In order to ensure the viability of the UCT after parturition and limit the microbiological contamination of the samples, the umbilical cords were transported from the hospital / clinic to the laboratory at refrigerated temperatures monitored by a datalloger in less than 72 hours. The UCT units from 15-20 centimeters-long umbilical cords and after the blood vessels dissection are treated and processed for cryopreservation using a cryoprotective solution (freezing medium). The UCT units are transferred to a computer-controlled slow rate freezer (Sylab, Consensus, Portugal) and a nine-step freezing program is used to set up the time, temperature, and rates specifically optimized for the human umbilical cord-MSCs cooling. To thaw frozen cells, the cryovials are transferred directly to a 37°C water bath. Upon thawing in less than a minute, the cell suspension is centrifuged at 150 × g for 10 min, and the supernatant is gently removed and the cell pellet is resuspended in culture medium. It was possible to obtain MSCs in culture from 52 out of 60 thawed UCT units.

In some UCT cryopreserved units (N = 8) it was not possible to isolate MSCs due to increase number of erythrocytes' lysis or microbiological contamination during cell culture. The MSCs morphology was observed in an inverted microscope (Zeiss, Germany) at different points of expansion. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. The MSCs obtained were characterized by flow cytometry (FACSCalibur®, BD Biosciences) analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). In the presence of neurogenic medium, the MSCs were able to, became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches. These results permitted to conclude that the processing and cooling protocols used for UCT units' cryopreservation were adequate to preserve the UCT viability since it was possible to isolate and expand MSCs after appropriate thaw and in presence of adequate cell culture conditions.

An established and ready-to-use Human MSC cell line was also employed for promoting axonotmesis and neurotmesis lesions regeneration. Human MSCs from Wharton's jelly umbilical cord were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreservated cells are cultured and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Mesenchymal Stem Cell Medium (PromoCell, C-28010) is replaced every 48 hours. At 80-90% confluence, cells are harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. MSCs at a concentration of 2500 cells/ml are cultured on poli-D-lysine coverslips (Sigma) or on biomaterials membranes and after 24 hours cells exhibit 30-40% confluence. Differentiation into neuroglial-like cells is induced with MSC neurogenic medium (Promocell, C-28015). Medium is normally replaced every 24 hours during 3 days. The formation of neuroglial-like cells can be observed after 24 hours in an inverted microscope (Zeiss, Germany) (Figure 5 and Figure 6).

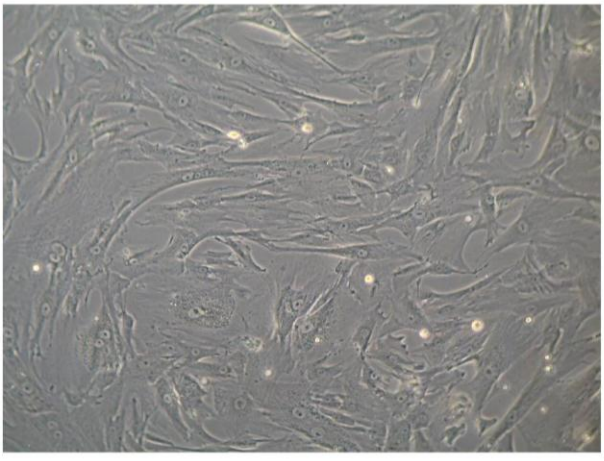

Figure 5. MSC cell line from Wharton's jelly (PromoCell) exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.

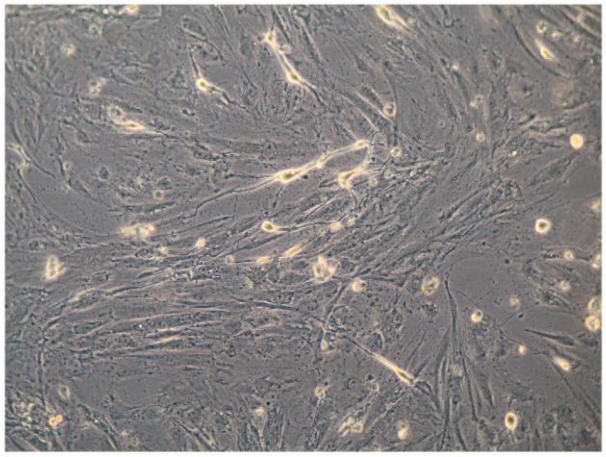

Figure 6. MSC cell line from Wharton's jelly (PromoCell) after 72h of incubation in neurogenic medium. The cells became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches. Magnification: 100x.

This established human MSC cell line is preferred for *in vivo* testing in rats, since the number of MSCs obtained is higher in a shorter culture time, it is not dependent on donors availability and ethic committee authorization, and the protocol is much less time consuming which is advantageous for pre-clinical trials with a large number of experimental animals. As a matter of fact, there is no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure. The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell MSCs isolated from Wharton's jelly of umbilical cord. MSCs are tested for cell morphology, adherence rate and viability. Furthermore, each cell lot is characterized by flow cytometry analysis for a comprehensive panel of markers.

The MSCs isolated with the two protocols described (from fresh and the cryopreserved UCT units) and from the established Promocell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion the cells became long spindle-shaped and colonized the whole culturing surface. After 96 hours of culture in neurogenic medium, cells changed in morphology. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches. Giemsa-stained cells of differentiated MSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations were found demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures [55, 56]. The differentiated MSCs karyotype could not be established, since no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated MSCs previously determined, excluded the presence of neoplastic cells,

thus supporting the suitability of our cell culture and differentiation procedures. This concern also resulted from our previous experience with N1E-115 neoplastic cell line and the negative results we obtained in the treatment of axonotmesis and neurotmesis injuries [57-59]. Nevertheless, undifferentiated MSCs from the Wharton's jelly culture (obtained from either protocol or from the Promocell cell line) showed normal morphology when inspected with an inverted microscope (Figure 7).

The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained from MSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN. After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN showing that differentiation of MSCs in neural-like cells was successfully achieved for MSCs obtained from UCT (fresh and cryopreserved) and for the Promocell MSC cell line (Figure 8) [55].

2.1.2. Differentiation into neuroglial-like cells

MSCs express nestin, a maker for neural and other stem cells [60, 61] and can be differentiated in adipose tissue, bone, cartilage, skeletal muscle cells, cardiomyocyte-like cells, and neuroglial-like cells [54, 55, 60, 62], presenting great potential to biomedical engineering applications. These cells fit into the category of primitive stromal cells and because they are abundant and inexpensive, they might be very useful for regenerative medicine and biotechnology applications.

By employing neuron-conditioned media, sonic hedgehog and fibroblast growth factor 8, MSCs isolated from the Wharton's jelly can be induced toward dopaminergic neurons. These cells have been transplanted into hemiparkinsonian rats where they prevented the progressive degeneration/behavioral deterioration seen in these rats [63]. Rat MSCs isolated from the Wharton's jelly when transplanted into brains of rats with global cerebral ischemia significantly reduced neuronal loss, apparently due to a rescue phenomenon [64]. Neuronal differentiation of human MSCs could also provide cells to replace neurons lost due to neurodegenerative diseases. Recent studies showed that transplanted MSCs-derived neurons become electrophysiologically integrated within the host neural tissue [65]. However, all these therapeutic applications need uniform and reproducible regulation.

A consequence of cell metabolism during *in vitro* expansion is that culture conditions are constantly changing. The comprehension and optimization of the expansion and differentiation process will contribute to maximization of cell yield, reduced need of cell culture, and a decrease in total processing costs [66, 67]. Elucidation of regulatory mechanisms of MSCs differentiation will allow optimization of *in vitro* culture and their clinical use in the treatment of neural-related diseases. Research is being performed to optimize expansion process parameters in order to grow MSCs in a controlled, reproducible, and cost-effective way [68]. Metabolism is certainly one of these parameters.

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3. Regeneration and in vivo testing

With the world wide global increase in life expectancy, a variety of disabling diseases with large impact on human population are arising. This includes cardiovascular, neurological, musculoskeletal, and malignancies. Therefore, it is imperative that new and more effective treatment methods are developed to correct for these changes. Further research with experimental animal systems is required to translate to in vivo cell-based therapy that has been extensively investigated in vitro [1]. Stem cell biology is probably the golden key for cell therapies and regenerative medicine. Regeneration is the physical process where remaining tissues organize themselves to replace missing or injured tissues *in vivo* [39].

It has been speculated that once MSCs have the potential to differentiate into several tissues, they might be responsible for turnover and maintenance of adult tissues, just like hematopoietic stem cells have this role in blood cells [69]. First, it was believed that after injection of MSCs, these were able to migrate to the damaged site and to differentiate into ones with the appropriate function for repairing, so MSCs could mediate tissue repair through there multilineage capacity replacing damaged cells. Subsequent studies have suggested that the mechanism used by MSCs for tissue repairing is not really this way. This new idea was reinforced by the confirmation that this cells homed to damaged site, particularly to spots of hypoxia, inflammation and apoptosis [70, 71].

Recent studies demonstrated that transplanted MSCs modified the surrounding tissue microenvironment, promoting repair with functional improvement by secretion factors (known as paracrine effect), stimulation of preexisting stem cells in the original tissue and decreasing of inflammation and immune response [72]. Other studies have demonstrated that MSC-conditioned media by itself could have therapeutic effects. All this data suggest that MSC apply a reparative effect on injured side through its paracrine effects [73].

It is necessary to overcome some barriers before a cell-based therapy becomes routine in clinics, including the cell number and the administration way of treatment. MSCs are difficult to be maintained stable in culture for long time, but due to their short doubling time, if at the outset many cells are harvested they may be properly scaled up in primary culture, never forgetting the ideal seeding number [39].

MSCs are an attractive candidate for cell-based regenerative therapy; the evidence is that currently there are 139 trial registries for MSC therapy 27 of which are based on umbilical cord MSCs [74].

3.1. Nerve regeneration

After Central Nervous System (CNS) lesions, Peripheral Nervous System (PNS) injuries are the ones with minor successes in terms of functional recovery. These kinds of injuries are frequent in clinical practice. About two centuries ago it was assumed that these nerves would never regenerate. Indeed, scientific and clinical knowledge greatly increased in this area. Nevertheless, a full understanding of axonal recovery and treatment of nerve defects,

especially complete functional achievement and organ reinnervation after nerve injury, still remains the principle challenge of regenerative biology and medicine [75, 76].

3.1.1. Nerve repair

Many peripheral nerve injuries can only be dealt through reconstructive surgical procedures. Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery, as functional recovery is rarely satisfactory in these patients [76]. Direct repair should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a nerve gap, are considered for a nerve graft procedure. In these cases, the donor nerves used for grafting are commonly expendable sensory nerves. This technique, however, has some disadvantages, with the most prominent being donor site morbidity, that may lead to a secondary sensory deficit and occasionally neuroma and pain. In addition, no donor and recipient nerve diameters often occurs which might be the basis for poor functional recovery. Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy, and entubulation by means of autologous non-nervous tissues, such as vein and muscles [76]. One advantage of these allografts compared with the autografts is the absence of donor site morbidity and theoretically the unlimited length of tissue available [77]. Experimental work from a number of laboratories has emphasized the importance of entubulation for peripheral nerve repair to manage nerve defects that cannot be bridged without tension (neurotmesis with loss of nerve tissue). Nerves will regenerate from the proximal nerve stump towards the distal one, whereas neuroma formation and ingrowth of fibrous tissue into the nerve gap are prevented [78]. The reliability of animal models is crucial for PN research, including therapeutic strategies using biomaterials and cellular systems. As a matter of fact, rodents, particularly the rat and the mouse, have become the most frequently used animal models for the study of peripheral nerve regeneration because of the widespread availability of these animals as well as the distribution of their nerve trunks which is similar to humans [79]. Because of its PN size, the rat sciatic nerve has been the most commonly experimental model used in studies concerning the PN regeneration and possible therapeutic approaches [80]. Functional recovery after PN injury is frequently incomplete, even with adequate microsurgery, so, many research and clinical studies have been performed including biomaterials for tube-guides. Since the 80's, Food and Drug Administration (FDA) has approved a variety of these biomaterials both natural and synthetic. The ideal biomaterial nerve graft should increase number, length and speed of axon regeneration [77]. It should be:

i. biocompatible, not toxic neither present undesired immunologic response;

ii. permeable enough to permit nutrient and oxygen diffusion and allows cell support systems;

iii. flexible and soft to avoid compression;

iv. biodegradable, the ideal rate is to remain intact during axon regeneration across nerve gap and after degrade softly and

v. technically reproducible, transparent, easy to manipulate, and sterilize [81].

Currently 3 types of materials are available for nerve reconstruction: non-resorbable, natural resorbable and synthetic resorbable. Polyvinil alcohol hydrogel (PVA) is an example of a non-absorbable biomaterial. It combines water in similar proportions to human tissue, with PVA providing a stable structure easy to sterilize, which is a main advantage of this materials, but has some limitations such as: nerve compression and suture tension after regeneration due to its non-resorbable nature [77]. Collagen type I from humans or animals, is an example of a natural resorbable device, which has some advantages such as:

i. easy to isolate and purify,

ii. good adhesiveness for supporting cell survival an proliferation,

iii. has been proven to be highly biocompatible and support nerve regeneration *in vivo*.

On the other hand, offers some immune response requiring the use of immunosuppressive drugs or pre-treatment of the material before clinical use [77]. Poly (DL-lactide-ε-caprolactone) (PLC) a synthetic resorbable material is the only transparent device approved by FDA, important characteristic for the surgeon that facilitates the insertion of the nerve stumps across the nerve gap, but, on the other hand it is not flexible [77]. Chitosan, PLC, collagen, poly(L-lactide) and poly(glycolide) copolymers (PLGA) and others, some of them, previously studied by our group [57, 58, 82] were associated to cellular systems, which are able to differentiate into neuroglial-like cells or capable of modulating the inflammatory process, improved nerve regeneration, in terms of motor and sensory recovery, and also shortening the healing period after axonotmesis and neurotmesis, avoiding regional muscular atrophy [57, 58, 82].

Researches with acellular nerve allografts, as alternative for repairing peripheral nerve defects have been reported. These nerve allografts remove the immunoreactive SCs and myelin however preserve the internal structure of original nerve, containing vital components such as collagen I, laminin and growth factors essential for repairmen of the lesions [83]. Acellular grafts remain insufficient, due to the increasing extent of nerve damages. Also, viable cells are necessary for debris removal and environmental regeneration reestablishment [83].

Cell transplantation, such as Schwann cells (SCs) transplantation has been proposed as a method of improving peripheral nerve regeneration [84]. SCs are peripheral glial cells that enwrap axons to form myelin with a central role in neuronal function. When there is damage in PNS, SCs are induced to mislay myelin, proliferate and segregate numerous factors, including cytokines responsible for reproducing a microenvironment suitable for supporting axon regeneration [85, 86]. They also have a vital participation in endogenous repair, reconstructing myelin, which are essential for functional recovery [85, 86]. SCs, MSCs, ESCs, marrow stromal cells are the most studied support cells candidates. SCs transplantation enhance axon outgrowth both *in vitro* [87] and *in vivo* [88]. Although to achieve an adequate amount of autologous SC, a donor nerve is necessary and a minimum of 4-8 weeks for *in vitro* expansion. Umbilical cord MSCs may be the perfect cell model as supplement for nerve grafts, once they are easily obtained, with no ethical controversy and can differentiate into

neuglial-like cells [83]. Matuse and collaborators induced MSCs from the umbilical cord into SCs capable of supporting peripheral nerve regeneration and myelin reconstruction *in vivo*. They transplanted these SCs into injured sciatic nerve, and proved that these cells maintained their differentiated phenotype *in vivo*, and contributed for axonal regeneration and functional recovery [89].

In our studies we aimed to explore the therapeutic value of human umbilical cord matrix (Wharton's jelly) derived MSCs, undifferentiated and differentiated in neuroglial-like cells, both *in vitro* and *in vivo*, associated to a variety of biomaterials such as, Poly (DL-lactide-ε-caprolactone) PLC (Vivosorb®) membrane, and Chitosan type III on rat sciatic nerve axonotmesis and neurotmesis experimental model. For cell transplantation into injured nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly inoculated into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively); or the cells can be pre-added to the neural scaffold via inoculation or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve [82].

Our PLC studies [55] demonstrated that this biomaterial does not interfere negatively with the nerve regeneration process, in fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. PLC becomes hydrophilic by water uptake, which increases the permeability of the polymer. This is essential for the control of nutrient and other metabolite transportation to the surrounding healing tissue. A few weeks after implantation, the mechanical power gradually decreases and there is a loss of molecular weight as a result of the hydrolysis process. Nearly in 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PCL has the advantage of not creating an acidic and potentially disturbing micro-environment, which is favorable to the surrounding tissue [90]. Chitosan has attracted particular attention in medical areas due to its biocompatibility, biodegradability, and low toxicity, low cost, improvement of wound-healing and antibacterial properties. Moreover, the potential use of chitosan in nerve regeneration has been demonstrated both *in vitro* and *in vivo* [57, 91]. Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin [57, 82]. While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups. Finally, the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. By adding GPTMS and employing a freeze-drying technique, we have previously obtained chitosan type III membranes (hybrid

chitosan membranes) with pores of about 110 μm diameter and about 90% of porosity, and which were successful in improving sciatic nerve regeneration after axonotmesis and neurotmesis [56, 57, 82].

The induction of a crush injury in rat sciatic nerve provides a very realistic and useful model of damage for the study of the role of numerous factors in regenerative processes [57]. Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After axonotmesis injury regeneration is usually successful, after a short (1-2 day) latency, axons regenerate at a steady rate towards the distal nerve stump, supported by the reactive SCs and the preserved endoneural tubules enhance axonal elongation and facilitate adequate reinnervation [92]. Our research group has been testing the efficacy of combining biomaterials and cellular systems in the treatment of sciatic nerve crush injury [57-59, 82, 90, 91, 93-95]. Following transection, axons show staggered regeneration and may take substantial time to actually cross the injury site and enter the distal nerve stump [60]. Although delayed axonal elongation might be caused by growth inhibition originating from the distal nerve itself, growth-stimulating influences may overcome axons stagger. More robust and fast nerve regeneration is expected to result in better reinnervation and functional recovery. As a potential source of growth promoting signals, MSCs transplantation is expected to have a positive outcome. Our results showed that the use of either undifferentiated or differentiated HMSCs enhanced the recovery of sensory and motor function in axonotmesis lesion of the rat sciatic nerve [56]. Neurotmesis must be surgically treated by direct end-to-end suture of the two nerve stumps or by a nerve graft harvested from elsewhere in the body in case of tissue loss. To avoid secondary damage due to harvesting of the nerve graft, a tube-guide can be used to bridge the nerve gap. Acutely after sciatic nerve transection there is a complete loss of both motor and thermal sensory function. Sensory and motor deficit then progressively decrease along the post-operative. From a morphological point of view, nerve regeneration occurs if Wallerian degeneration is efficient and is substituted by re-growing axons and the accompanying viable SCs [96, 97]. The axon regeneration pattern is improved by using appropriate biomaterials for the tube-guide design, like chitosan type III and PLC and cellular systems like MSCs from the Wharton jelly [57, 90, 91, 95]. The surgical technique and the time for the reconstructive surgery is also crucial for the nerve regeneration after neurotmesis [57, 90, 91, 95].

3.2. Assessment of nerve regeneration in the sciatic nerve rat model

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [94, 98-100]. Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling [79] do not necessarily predict the reestablishment of motor and sensory functions [100-103]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [100]. In this sense, research on peripheral nerve injury needs to combine both functional and morphological assessment. The use of biomechanical techniques and rat's gait kinematic evaluation is a prog-

$$\text{Print length factor } (\text{PLF}) = (\text{EPL} - \text{NPL}) / \text{NPL} \quad (4)$$

SFI is calculated as described by Bain et al. [108] according to the following equation:

$$\begin{aligned} \text{SFI} &= -38.3(\text{EPL} - \text{NPL}) / \text{NPL} + 109.5(\text{ETS} - \text{NTS}) / \text{NTS} + 13.3(\text{EIT} - \text{NIT}) / \text{NIT} - 8.8 \\ &= (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \end{aligned} \quad (5)$$

For SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given [109]. In each walking track 3 footprints are analyzed by a single observer, and the average of the measurements is used in SFI calculations.

Ankle kinematics analysis is carried out prior nerve injury, at week-2 and every 4 weeks during the 12 or the 20-week follow-up time, for axonotmesis and neurotmesis lesions, respectively. The motion capture is performed with 2 digital high speed cameras (Oqus, Qualysis®) at a rate of 200 images per second, and Qualisys Track Manager software (QTM, Qualysis®). The cameras operate on a infra-red light frequency ensuring a high level of accuracy on the determination of reflective marker position and a position residual of less than 2.7 mm was obtained. Cameras are usually positioned to not recorder significant signal deflection during the test and four reflective markers were placed at the skin of the rat right hindlimb at the proximal edge of the tibia, the lateral malleolus and the fifth metatarsal head. Advanced analysis of the 2-D movement (sagittal plan) data is performed with Visual3D software (C-Motion®, Inc). The rats' ankle angle is determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint ($\theta°$) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points are identified: midswing, midstance, initial contact (IC) and toe-off (TO) [104, 109-113] and are time normalized for 100% of step cycle. The normalized temporal parameters are averaged over all recorded trials. Angular velocity of the ankle joint (Ω °/s) is also determined where negative values correspond to dorsiflexion. A total of 6 walking trials for each animal with stance phases lasting between 150 and 400 ms are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) [104]. Animals walk on a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments.

3.2.2. Morphologic Assessment

Nerve samples are processed for quantitative morphometry of myelinated nerve fibers [114]. Fixation is usually carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding is obtained following Glauerts' procedure (Scipio et al., 2008). Series of 2-μm thick semi-thin transverse sections are cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany)

and stained by Toluidine blue. Stereology is carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector is always adopted using a protocol previously described [115, 116]. Fiber density and total number of myelinated fibers is estimated together with fiber and axon diameter and myelin thickness.

3.3. Results

3.3.1. Differentiation and metabolism of MSCs from Wharton's jelly

In our experimental studies we expanded undifferentiated MSCs from human umbilical cord Wharton's jelly that exhibited a normal star-like shape with a flat morphology in culture (Figures 4 and 5). To prevent the possibility of eventual mutations due to expansion artifacts, a total of 20 Giemsa-stained metaphases of these cells, were analyzed for numerical aberrations. Sporadic, non-clonal aneuploidy was found in 3 cells (41-45 chromosomes). The other 17 metaphases had 46 chromosomes (Figure 7). The karyotype was determined in a completely analyzed G-banding metaphase. No structural alterations were found. The karyotype analysis to the MSCs cell line derived from Human Wharton jelly demonstrated that this cell line has not neoplastic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes [55].

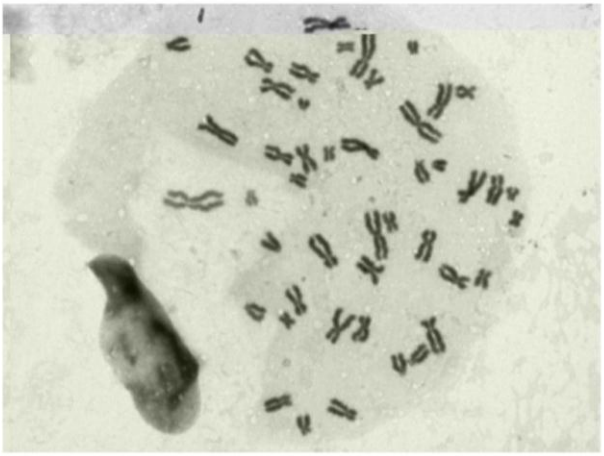

Figure 7. Selected metaphases from undifferentiated MSC cells isolated from Wharton's jelly, showing the normal number of chromosomes (46, XY). Magnification: 1000X.

We differentiated MSC from Wharton's Jelly into neuroglial-like cells. After 96 hours of incubation in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neural-like cells with multi-branches and secondary branches (Figure 6). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained

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from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN (Figure 8A,C,E). After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP (Figure 8B) and for the growth-associated protein GAP-43 (Figure 8D). All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN (Figure 8F) showing that differentiation of MSCs in neural-like cells were successfully achieved [55].

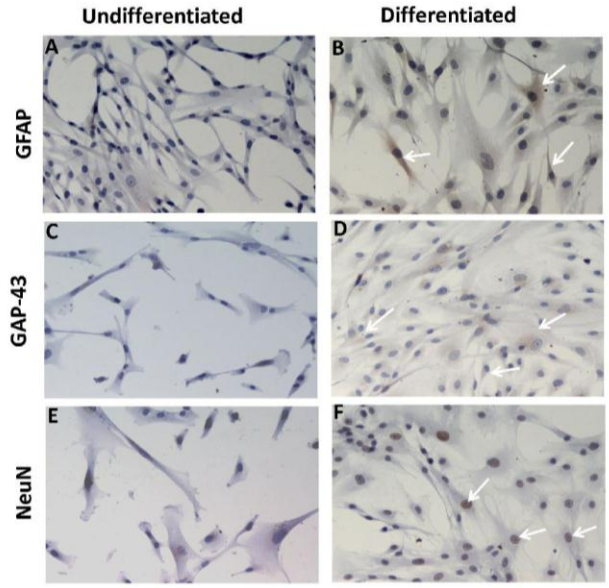

Figure 8. Undifferentiated MSC cells from the Wharton's jelly presenting a negative staining for: (A) GFAP which is a glial cell marker; (C) GAP-43 which is related with axonal outgrowth and (E) NeuN which is a marker for nucleus of neurons. Neuroglial-like cells obtained from HMSCs in vitro differentiated with neurogenic medium exhibiting a positive staining for: (B) GFAP; (D) GAP-43 and (F) NeuN. Magnification: 200x [55].

The *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization. Although pre-clinical and clinical data demonstrated the safety and effectiveness of MSCs therapy in some pathologies such as neurological, there are still questions surrounding the mechanism of action. In our research work we aimed to disclose the possible role of metabolism not only in the MSCs maintenance and expansion but also during the differentiation in neural-like cells [55]. MSCs maintenance and differentiation, to neural-like cells, depends on metabolic modulation. *In vitro*, glucose is the most widely used substrate for the generation ATP which is essential for cell growth and maintenance. It has been proposed that cells undergoing high proliferation rates depend on glycolysis to generate ATP, known as Warburg effect, although this pathway is less effective than the oxidative phosphorylation in terms of ATP production [117]. Our results showed that during expansion, the undifferentiated MSCs consume glucose and produce high concentration of lactate as a metabolic sub product which is consistent with the

Warburg effect and glycolysis stimulation. MSCs do not require oxidative phosphorylation to survive as alternative, hypoxia extends the lifespan, increases their proliferative ability and reduces differentiation [118]. The morphologic and biochemical characteristics of neural-like cells are already described but the mechanism by which stem cells differentiate into neural-like cells is still unknown. In our research work, MSCs that undergone differentiation into neural-like cells, consumed significantly less glucose and produced significantly less lactate than MSCs that undergone only expansion. These major differences allow us to conclude that during MSCs differentiation in neural-like cells the glycolytic process, which proved to be the crucial metabolic mechanism during MSCs expansion, is switched to oxidative metabolism [55].

Our results show clear evidences that MSCs expansion is dependent of glycolysis while their differentiation in neural-like cells requires the switch of the metabolic profile to oxidative metabolism. Also important may be the role of oxidative stress during this process. This work is a first step to identify key metabolic-related mechanisms responsible for human MSCs from the Wharton's jelly expansion and differentiation [55].

The lack of standardization of MSCs isolated from the Wharton's jelly culture conditions has limited some progress in scientific and clinical research. Understanding these MSCs metabolism during expansion, as well as determining molecular and biochemical mechanisms for differentiation is of great significance to develop new effective stem cell-based therapies.

4. Biomaterial and cellular system association – discussion and final remarks

Using the rat model, we recently tested *in vivo* the efficacy of biomaterials and cellular system association in treatment of sciatic nerve axonotmesis and neurotmesis injury. Following transection, axons show staggered regeneration and may take substantial time to cross the injured site and enter the distal nerve stump [119]. However delayed axonal elongation might be caused by growth inhibition originated from the distal nerve itself, growth-stimulating influences may overcome axons stagger. As a potential source of growth promoting signals, MSCs transplantation is expected to give a positive outcome. Our results showed that the use of either undifferentiated or differentiated MSCs in axonotmesis lesion boosted the recovery of sensory and motor function. In both cell-enriched experimental groups we observed that the myelin sheath was thicker, this suggests that MSCs might apply their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. Also results from *in vivo* testing previously performed by our research group showed that infiltration of MSCs from the Wharton's jelly, or the combination of chitosan type III membrane enwrapment and MSCs enrichment after nerve crush injury provide an advantage to post-traumatic nerve regeneration [56, 57]. Chitosan type III was developed as a hybrid of chitosan by adding GPTMS. A synergistic effect of an extra permeability and physicochemical properties of chitosan type III and the presence of silica ions

may be responsible for the good results in post-traumatic nerve regeneration promotion observed in the sciatic nerve after axonotmesis and neurotmesis [57, 91]. The substantial improvement of axonal regeneration found in sciatic nerve crush enwrapped by chitosan type III membranes and for bridging nerve gaps after neurotmesis [57, 91], suggests that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration. The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation and myelinization [55, 91]. Our data also showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. The MSCs from the Wharton's jelly may be a valuable source in the repair of the peripheral nervous system with capacity to differentiate into neuroglial-like cells. The transplanted MSCs are also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) [55]. Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplastic origin [57-59, 91, 93]. The MSCs isolated from the Wharton´s jelly through PLC and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. The results from our experimental work [55, 56] showed that the use of either undifferentiated or neuroglial-like differentiated MSCs enhanced the recovery of sensory and motor function of the rat sciatic nerve. The observation that in both cell-enriched experimental groups myelin sheath was thicker, suggest that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used) of the UC units from the hospital / clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved UCT. It is highly recommend that the transport from the clinic or hospital to the laboratory should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

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6. Methods for functional recovery analysis after axonotmesis and neurotmesis

Even after all these studies, the functional outcome in patients still remains limited. The results and the understanding of such limitation are greatly dependent on the approaches, methods and therapeutic strategies. Choosing the correct experimental model and functional assay for peripheral nerve injury is essential. After peripheral nerve injury there are neural and mechanical disorders which are the major limitations to study the functional meanings of recovery (Nichols *et al.*, 2005). The rat has become the most appellative animal model in peripheral nerve regeneration studies. Primarily due to the large availability of these animals, and also because of the similarity to humans in the distribution of their nerve trunks (Mackinnon *et al.*, 1985). Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing tool for injuries comprising plurifascicular mixed nerves. The rat sciatic nerve is the most experimented model since it provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and application of grafts or nerve conduits (Mackinnon *et al.*, 1985). To evaluate functional recovery after sciatic nerve injury there are several methods available that have been extensively used by our research group.

6.1. Extensor postural thrust

The Extensor Postural Trust (EPT) or motor reflex function was originally proposed by Thalhammer and collaborators in 1995, for the calculation of part of the neurological recovery in rats after sciatic nerve injury (Thalhammer *et al.*, 1995). In this neurological test, the totality of the rat's body, with exception of the hind limbs, is wrapped in a surgical towel and supported by the thorax (Figure 11). The affected hind limb is then let down towards the platform of a digital balance to produce the EPT. As the animal is lowered over the platform, he extends the hind limb, anticipating the contact made by the distal metatarsus and digits. The force applied (in a scale range 0-500 g) to the digital platform balance is recorded in grams (Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b).

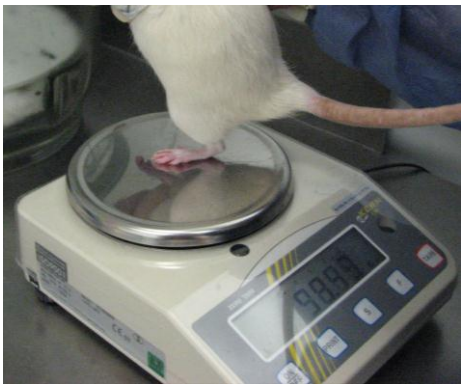

Figure 11 - Extensor Postural Trust (EPT) neurological test.

The reduction in this force, representing a reduced extensor muscle tone, is considered a deficit of motor function. The same procedure is applied to the contra-lateral, unaffected limb. The affected and normal limbs are tested 3 times, with an interval of 2 minutes between consecutive trials, and the three values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b).
The percentage of functional deficit (range between 0 and 1), is derived from Equation (1) as described in the literature by Koka and Hadlock (Koka *et al.*, 2001):

$$Motor\ Deficit = \frac{NEPT - EEPT}{NEPT} \qquad \text{(Equation 1)}$$

6.2. Withdrawal Reflex Latency

The Withdrawal Reflex Latency (WRL) or hot plate sensory test is a model that allows independent motor and sensory testing. The rat is wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hotplate at 56ºC (Figure 12). WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw. Normal rats withdraw their paws from the hotplate within 4 seconds or less (Hu *et al.*, 1997). The affected and normal limbs are tested three times, with an interval of 2 minutes between consecutive testing to prevent sensitization. The mean value is calculated for the three latency times to obtain a final result. The maximum time for heat stimulation is set at 12 seconds to avoid skin damage to the foot (Varejão *et al.*, 2003a; Varejão *et al.*, 2003b; Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b).

Figure 12 - Withdrawal Reflex Latency (WRL) sensory test (Luís, 2008).

6.3. Sciatic Functional Index and Static Sciatic Index

Normal function of the hind limb in the rat is crucial for an accurate gait and posture. After a nerve injury posture and function of leg, foot and toe will be affected, changing the patterns of paw stepping and placement (de Medinaceli *et al.*, 1982; Nichols *et al.*, 2005). Sciatic Functional Index (SFI) is a method in which animals are tested in a confined walkway measuring 42-cm long and 8.2-cm wide, with a dark shelter at the end. A white paper is placed on the floor of the rat walkway. The hind paws of the rats are pressed down onto paint soaked sponge after what they are allowed to walk down the walkway leaving its hind footprints on the paper. Often, several walks are required to obtain clear print marks of both feet. Prior to any surgical procedure, all rats are trained to walk in the walkway, and a baseline walking track is recorded. Subsequently, walking tracks are recorded every week until the week-8 postoperatively and then every 2 weeks (Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b).

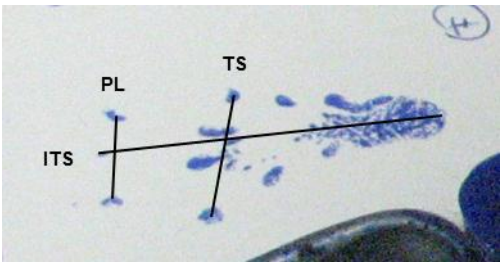

Figure 13 – Measurements taken from the footprints for calculation of SFI value.

Several measurements are taken from the footprints (Figure 13):

(i) distance from the heel to the third toe, the print length (PL);

(ii) distance from the first to the fifth toe, the toe spread (TS); and

(iii) distance from the second to the fourth toe, the intermediary toe spread (ITS).

The mean distances of the measurements are used to calculate the following factors SFI and SSI (dynamic and static), where the capital letters E and N indicate injured (experimental) and non-injured side (normal), respectively.

$$Toe\ spread\ factor\ (TSF) = \frac{ETS - NTS}{NTS} \quad \text{(Equation 2)}$$

$$Intermediate\ toe\ spread\ factor\ (ITSF) = \frac{EITS - NITS}{NTIS} \quad \text{(Equation 3)}$$

$$Print\ length\ factor\ (PLF) = \frac{EPL - NPL}{NPL} \quad \text{(Equation 4)}$$

SFI is calculated as described by Bain et al. (Bain *et al.*, 1989) according to the following equation:

$$SFI = \frac{(EPL - NPL)}{NPL} + 109.5\frac{(ETS - NTS)}{NTS} + 13.3\frac{(EIT - NIT)}{NIT} - 8.8 = \\ = (-38.3\ \text{x PLF}) + (109.5\ \text{x TSF}) + (13.3\ \text{x ITSF}) - 8.8 \quad \text{(Equation 5)}$$

Static footprints are obtained at least during four occasional rest periods. For the sciatic static index (SSI) only the parameters TS and ITS, are measured (Bervar 2000):

$$SSI = [(108.44\ \text{x TSF}) + (31.85\ \text{x ITSF})] - 5.49 \quad \text{(Equation 6)}$$

For both SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given (Dijkstra *et al.*, 2000).

6.4. Kinematic analysis of the rat gait

As mentioned before the selection of a good functional assay is of extreme importance. Nevertheless after neural injury there are other dysfunctions that must be evaluated, and for that reason the analysis of the locomotion is of extreme importance. A considerable work has been developed in order to establish methods for locomotion analysis in animal models. Kinematic parameters are essential tools for the evaluation of functional nerve

recovery. The first system to be used for locomotion function analysis was the open field locomotion scoring system formulated by Tarlov and Klinger in 1954. After this, many reformulations to this scoring system were done (Couto *et al.*, 2008). With the advances in high technology, instrumented rat gait methods have been highly used in research. Voluntary movement and reflex activity are considered for functional assay analysis. Gait functions represent the combination of motor and sensory function and several gait parameters are considered with significant relationship for functional evaluation of experimental rat model.

The kinematic two dimensional (2D) approach is the most friendly, since it is simple and requires only one camera to record the step cycles (Couto *et al.*, 2008). Locomotion is described by a repetitive sequence of limb motion to move the body forward while maintaining the stance balance. There are three basic approaches to analyse the gait (Perry *et al.*, 1993): (i) subdivision of the cycle according to the variations in reciprocal floor contact by the two feet; (ii) using the time and distance qualities of the stride; and (iii) identifying the functional significance of the events within the gait cycle and designating these intervals as the functional phases of gait. According to the variations in reciprocal floor contact by the two feet, as the body moves forward, one limb serves as mobile source of support while the other limb advances itself to a new support site. Stance phase designates the entire period during which the foot is on the ground and begins with initial contact (IC) and Swing phase is the phase of the normal gait cycle during which the foot is off the ground. The swing phase follows the stance phase and is divided into (i) initial swing, (ii) midswing, and (iii) terminal swing stages. Analysis of the free walking pattern of the rat is the mostly used method for assessment of motor function through motion analysis. Although locomotor activity in an open field is a stable behavioural test and may be used as an index of the behavioural-physiological coping style of an individual rat (Basso, Beatie and Bresnahan (BBB) Locomotor Rating Scale), the information obtained is qualitative, ranging from 0 to 21 and distinguishing between locomotors features such as flaccid paralysis, isolated hind-limb joint movements, weight-supported plantar stepping, coordination, and details of locomotion (eg, toe clearance, paw position) (Basso *et al.*, 1995; Basso, 2000).

Ankle kinematics is carried out prior to nerve injury (week 0), and at the end of the follow-up time. Animals walk on a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments. The rats' gait is recorded at a rate of 300 Hz images per second. The camera is positioned at the track

half length, where gait velocity is stable, and 1 m distant from the track obtaining a visualization field of 14 cm wide. The video images are stored for later analysis using an appropriate software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, USA). 2-D biomechanical analysis (sagittal plan) is carried out applying a two-segment model of the ankle joint, adopted from the model firstly developed by Varejão et.al. (Varejão *et al.*, 2004). The rats' ankle angle is determined using the scalar product between two vectors representing the foot and the lower leg. With this model, positive and negative values of position of the ankle joint (θ^{o}) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points are identified: initial contact (IC), Opposite Toe off (OT), and Heel Rise (HR) and toe-off (TO) and are time normalized for 100% of step cycle (Dijkstra *et al.*, 2000; Varejão *et al.*, 2002; Varejão *et al.*, 2003b). The normalized temporal parameters are averaged over all recorded trials. A total of six walking trials for each animal with stance phases lasting between 150 and 400 ms (milliseconds) are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) (Varejão *et al.*, 2003b).

7. Morphological analysis

It is known that morphological analysis is the most common method for peripheral nerve regeneration studies. Actually, the investigation of nerve morphology can give us important information on various aspects of the regeneration processes which relate with nerve function (Geuna *et al.*, 2009; Raimondo *et al.*, 2009). Quantitative calculation of nerve fibres is an essential element to evaluate the effectiveness of experimental microsurgical techniques in the repair of severed peripheral nerves. The data obtained from histomorphometric analysis like number, density, and size of nerve fibres can provide answers to several important questions, about a new microsurgical technique (Geuna *et al.*, 2004). Assessment of myelinated nerve fibre-size in peripheral nerves is a common element used in peripheral nerve regeneration research (Geuna *et al.*, 2001). Yet, nerve function cannot be evaluated using just one parameter by itself (Dellon et al. 1989; Kanaya *et al.*, 1996; Geuna *et al.*, 2001), the association of some morphological parameters with functional recovery of a repaired nerve has been demonstrated, consolidating the importance of the morpho-quantitative assessment in the context of experimental nerve studies. The size of a myelinated nerve fibre is measured by several geometrical parameters: the diameter, the perimeter and the cross-sectional area. When high-resolution optical observation is the same parameters can be measured for the axon,

allowing the calculation of myelin thickness (Geuna *et al.*, 2001). It takes about 2-3 hours to estimate the density and total number of myelinated fibres in one single nerve (Geuna *et al.*, 2000). Therefore an efficient time-saving method is required.

The method used in this work (designed-based sampling strategy) was developed by Geuna and colleagues, it was designed in a fashion to apply stereological principles to peripheral nerve quantitative morphology by estimation of the density and total number of myelinated fibres (Geuna, 2000; Geuna *et al.*, 2000). Designed-based sampling strategies permits that all objects in the sampling space have an equal opportunity of being sampled, therefore these is a probability-based sampling strategy (Geuna, 2000). This sampling design requires a large amount of sampling in order to obtain a precise estimation. Here the fibre tops are used as sampling object instead of the profiles due to the morphological variability especially in size, shape and orientation. The dissector principle is used for the sampling of myelinated nerve fibres in peripheral nerves in order to obtain unbiased estimates of fibre density and total number. The method allows us to identify the top of fibres on two-dimensional (2-D) transverse sections of a nerve (Geuna, 2000). In the experimental work here described the estimation of, the total number of myelinated fibres, the mean diameter of fibre and axon of the regenerated and control nerve fibres was done.

Chapter II

Aims

Aims

As mentioned before, sciatic nerve injuries themselves are rare in humans; nevertheless this experimental model provides a very realistic testing tool for injuries involving plurifascicular mixed nerves. The rat sciatic nerve is the most experimented model since it provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and application of grafts or nerve conduits (Mackinnon *et al.*, 1985).

Mesenchymal stem cells (MSCs) have become one of the most seducing targets for tissue regeneration due to their striking characteristics; high plasticity, proliferative and differentiation capacity together with their attractive immunosuppressive properties. MSCs present low immunogenicity and high immunosuppressive properties once Human Leucocyte Antigen (HLA) class II expression is in very low levels or even absent. Research in this field has brought exciting promises in many disorders and therefore in tissue regeneration. The differentiation potential of MSCs in multilineage end-stage cells is already proven, and their potential for treatment of several diseases is well established (Gärtner *et al.*, 2013). The ability of MSCs to deliver trophic mechanisms, including secretion of cytokines that might serve both paracrine and endocrine functions have resulted in reduction of inflammation, apoptosis and fibrosis in numerous disease, transforming this cells into a valuable tool in regenerative medicine (Ankrum *et al.*, 2010). MSCs isolation can be performed from several different tissues, including bone marrow, adipose tissue, skeletal muscle, umbilical cord matrix and blood, placental tissue, amniotic fluid, synovial membranes, dental pulp, fetal blood, liver, and lung (Phinney *et al.*, 2007). MSCs isolated from the umbilical cord matrix (Wharton's jelly) present several advantages, such as shorter population doubling time, easy culture in plastic flasks, and good tolerance towards the immune system. As a matter of fact, local or systemic application of MSCs in non-immunosuppressed animals does not induce acute rejection which has been demonstrated in the experimental work discussed in this manuscript. In addition these cells have anticancer properties, and absence of tumorigenic activity, they are very easy to obtain and to isolate, and do not raise ethical controversies, since they are collected from tissues usually discarded at birth (Gärtner *et al.*, 2013). Therefore, this cellular system raised deep interest by our research group in order to study the therapeutic effect of MSCs associated to several developed and commercially available biomaterials in nerve regeneration promotion.

The rat sciatic nerve was the selected model to test the association of biomaterials with human MSCs (HMSCs) isolated from the umbilical cord Wharton's jelly. For the axonotmesis injury the goal was to test the therapeutic value of undifferentiated MSCs

associated or not to two different biomaterials, a hybrid chitosan membrane and a PLC membrane; and differentiated neuroglial-like MSCs in association with PLC (Table 1). In the first study, "*Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model",* a standardized axonotmesis injury was reconstructed with local application of undifferentiated MSCs associated or not to a hybrid chitosan membrane. The functional recovery was evaluated during 12 weeks by EPT and WRL tests and the morphological analysis was carried out with morphology and stereology analysis.

In the second study, *"Use of poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly of the umbilical cord for promoting nerve regeneration in axonotmesis: In vitro and in vivo analysis"*, the induced standardized axonotmesis injury was treated with undifferentiated and differentiated neuroglial-like MSCs associated with a PLC membrane. Also in this work, the functional recovery was evaluated during 12 weeks by EPT, WRL and SFI tests and the morphological analysis was carried out with stereology analysis.

Table 1 - Experimental groups performed for the axonotmesis injury study.

Injury	Group	Procedure	Follow-up Time	Publication
Axonotmesis	*Crush*	No treatment	12 weeks	1st,2nd
	CrushCell	Suspension HMSC	12 weeks	1st,2nd
	CrushChitIIICell	HMSC + Chitosan Type III membrane	12 weeks	1st
	CrushChitIII	Chitosan Type III membrane	12 weeks	1st
	CrushPLC	PLC membrane	12 weeks	2nd
	CrushCellNonDIFPLC	HMSC+PLC membrane	12 weeks	2nd
	CrushCellDIFPLC	Neural-like HMSC+PLC membrane	12 weeks	2nd

This type of injury (axonotmesis) is the most appropriate to investigate the cellular and molecular behaviour of peripheral nerve regeneration, and to accomplish preliminary *in vivo* testing of the cellular system and biomaterials used in tube-guide fabrication for more serious injuries of the peripheral nerve like neurotmesis. This particular work also permitted us to understand the clinical importance of the use of undifferentiated MSCs or differentiated into neuroglial-like cells.

Axonotmesis is a less severe lesion and has a good clinical prognosis and in most of the cases surgical reconstruction is not needed, nevertheless in the absence of an intervention, functional regeneration may not be complete, leading to muscle atrophy. It

was also our intention to study therapeutic approaches including the use of MSCs associated to different bio membranes, to improve the regenerative process in axonotmesis lesions to overcome the delayed regeneration frequently observed in this kind of injury leading to a poor functional recovery.

In the third published study "*Use of poly(DL-lactide-ε-caprolactone) membranes and Mesenchymal Stem Cells for promoting nerve regeneration in a Neurotmesis rat model: in vitro and in vivo analysis"*, we proposed to study the therapeutic value of undifferentiated and differentiated neuroglial-like MSCs associated to a PLC membrane in a more serious lesion of the peripheral nerve system that implies reconstructive microsurgery techniques – the neurotmesis. The functional recovery was evaluated during 20 weeks by EPT, WRL, SFI and ankle cinematic tests and the morphological analysis was carried out with stereology analysis.

At last and also for neurotmesis lesion, the aim of the study "*Effects of umbilical cord tissue mesenchymal stromal cells (UCX®) on rat sciatic nerve regeneration after neurotmesis injuries*.", was to evaluate the therapeutic value of human MSCs from the umbilical cord matrix either alone or administered with a ready-to-use commercial haemostatic matrix vehicle Floseal®, on functional and morphological recovery in chronic (20 week evolution), hyper-acute (3 days) and an acute phase (21 days) healing periods (Table 2).

The functional recovery was evaluated during 20 weeks by EPT, WRL, SFI and ankle cinematic tests and the morphological analysis was carried out with stereology analysis.

The cells used in the *in vivo* testing of 3 of the referred published papers were an established ready-to-use Human MSC cell line purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Established human MSC cell lines are preferred for *in vivo* testing, once the numbers of MSCs obtained are higher in a shorter culture time, therefore the study is not dependent on donors availability and ethic committee authorization, and the protocol is much less time consuming which is an advantageous for pre-clinical trials with a large number of experimental animals.

Table 2 - Experimental groups performed for Neurotmesis injury study.

Injury	Group	Procedure	Follow-up Time	Publication
Neurotmesis	*Neuro*	No treatment or repair	20 weeks	3rd, 4th
	End-to-end	End-to-end suture repair	20 weeks	3rd, 4th
	Graft	10mm autograft	20 weeks	3rd, 4th
	End-to-endPLCcellnonDif	End-to-end suture+PLC + HMSCs	20 weeks	3rd
	End-to-endPLCcellDif	End-to-end suture+PLC + Neural-like HMSC	20 weeks	3rd
	graftPLCcellnonDif	10mm autograft+PLC+HMSC	20 weeks	3rd
	graftPLCcellDif	10mm autograft+PLC+ Neural-like HMSC	20 weeks	3rd
	End-to-EndCMC	End-to-end suture+CMC	21 days	4th
	End-to-EndCMCMSCs	End-to-end suture+CMC+HMSC	21 days	4th
	End-to-EndMSCs	End-to-end suture +HMSC	21 days	4th
	Endto-EndFlosealMSCs	End-to-end suture+Floseal+HMSC	20 weeks	4th
	End-to-EndMSCs	End-to-end suture +HMSC	20 weeks	4th
	Endto-EndFloseal	End-to-end suture+Floseal	20 weeks	4th
	End-to-End	End-to-end suture	3 days	4th
	End-to-EndCMC	End-to-end suture+CMC	3 days	4th
	End-to-EndMSC	End-to-end suture +HMSC	3 days	4th
	End-to-EndCMCMSC	End-to-end suture+CMC+HMSC	3 days	4th
	End-to-EndFlosealMSC	End-to-end suture+Floseal+HMSC	3 days	4th
	End-to-EndFloseal	End-to-end suture+Floseal	3 days	4th

The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell MSCs isolated from Wharton's jelly of umbilical cord. MSCs are tested for cell morphology, adherence rate and viability. Furthermore, each cell lot is characterized by flow cytometry for a widespread marker panel.

For the last work the cell line used for the *in vivo* studies was a MSC cellular system (UCX®) established by a private company (ECBio - Research and Development in Biotechnology S.A.) that settled collaboration with our group for the *in vivo* testing of their cellular product. ECBio developed proprietary technology to isolate, expand, and cryopreserve a well-characterized population of human stromal cells derived from the umbilical cord tissue, Wharton's jelly, named herein as UCX® cells (Santos *et al.,* 2008).

UCX® cells phenotype, cell morphology, adherence rate and viability is in the overall the same as the MSC cell line used in the previous work.

In what concerns to *in vitro* testing of the cellular systems, several analysis were performed, before *in vivo application* to our animal model. Cytogenetic analysis was performed to guarantee that there was no chromosomal mutation occurring during cell culture procedures and immunocytochemistry was used to certify that differentiation of HMSCs into neuroglial-like cells was successfully performed.

Chapter III

Results

1. Axonotmesis injuries

2.1. Introduction

Axonotmesis is a well-studied nerve injury model, and the most appropriate to investigate the cellular and molecular behaviour of peripheral nerve regeneration. This permits to measure the role of the different factors and to accomplish preliminary *in vivo* testing with different therapeutic approaches (Fu *et al.*, 1997).

The axonotmesis approach used in this work, is performed after nerve mobilisation with a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria) exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3 mm lesion. This method allows to perform a standardized and reproducible lesion (Luís *et al.*, 2007a). The axonotmesis or crush injury is easy to be induced experimentally, with low costs and has a good regenerative capacity, identical in rats and sub-human primates (Varejão *et al.*, 2004). Crush injury causes axonal interruption but conserves the connective sheaths, the basal *laminae* of SCs are preserved, therefore allowing an optimal orientation and regrowth of the damaged axons (Amado *et al.*, 2008; Luís *et al.*, 2008b). After axonotmesis injury regeneration is generally successful, the reactive SCs and the preserved endoneurial tubules support axonal elongation along the distal nerve and assist an adequate reinnervation (Luís *et al.*, 2008b). The recovery time for this kind of injury has been highly studied (Varejão *et al.*, 2003c; Varejão *et al.*, 2004), and it was previously concluded that 8 weeks would not be enough as healing period for total functional and morphological recovery. Previous results showed that while a full recovery of nociception (WRL), SFI/SSI, and percentage of motor deficit (EPT) was achieved by weeks 5, 7 and 9, respectively, ankle kinematic parameters were still recovering their original values at week 12 post-operatively. The same being true for several morphoquantitative parameters of regenerated nerve fibres (Luís *et al.*, 2007a). Consequently Luís and colleges have implemented 12 weeks of healing recovery period to evaluate the functional and histomorphometric parameters recovery (Luís *et al.,* 2007a; Luís *et al.,* 2008b).

Crush injuries do not involve surgical reconstruction but due to the required regeneration period, neurogenic atrophy of the innervated muscles may occur, therefore, therapeutic approaches to successfully decrease this recovery time are of extreme importance. Tissue engineering association of biomaterials, like PLC or hybrid chitosan, to cellular systems, like MSCs able to differentiate into neuroglial-like cells,

might improve peripheral nerve regeneration, in terms of motor, sensory and histomorphometric parameters.

Chitosan has attracted particular attention due to its biocompatibility, biodegradability, and low toxicity, low cost, improvement of wound-healing and antibacterial effects (Amado *et al.*, 2008; Simões *et al.*, 2010). In addition, the potential utility of chitosan in nerve regeneration has been demonstrated both *in vitro* and *in vivo* (Senel *et al.*, 2004). In a previous study performed by our group, *in vivo* results showed that type III chitosan (hybrid chitosan) improved both morphological regeneration of nerve fibre and functional recovery (Amado *et al.*, 2008).

The MSCs may be directly injected into the neural scaffold which is enwrapped around the crush injury, or pre-added to the neural scaffold *via* injection or co-culture after which the biomaterial with the cellular system is implanted in the injured nerve. In the next two studies cells were added in both forms (Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b). MSCs are a potential source for peripheral nerve repair. Human MSCs from Wharton's jelly of the umbilical cord own stem cell properties (Yang *et al.*, 2008) and it has been demonstrated that human MSCs can differentiate into neuroglial-like cells (Fu *et al.*, 2006) or even modulate inflammatory process. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells which can be: harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses (Gärtner *et al.*, 2013).

MSCs isolated from the umbilical cord matrix (Wharton's jelly) demonstrate several advantages, such as shorter population doubling time, easy culture in plastic culture flasks, good tolerance towards the immune system, therefore transplantation into non-immunosuppressed animals does not induce acute rejection, anticancer properties, and most important absence of tumorigenic activity (Gärtner *et al.*, 2013).

1.2

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Special Issue

Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model****★●

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Abstract

Many studies have been dedicated to the development of scaffolds for improving post-traumatic nerve regeneration. The goal of this study was to assess the effect on nerve regeneration, associating a hybrid chitosan membrane with non-differentiated human mesenchymal stem cells isolated from Wharton's jelly of umbilical cord, in peripheral nerve reconstruction after crush injury. Chromosome analysis on human mesenchymal stem cell line from Wharton's jelly was carried out and no structural alterations were found in metaphase. Chitosan membranes were previously tested *in vitro*, to assess their ability in supporting human mesenchymal stem cell survival, expansion, and differentiation. For the *in vivo* testing, Sasco Sprague adult rats were divided in 4 groups of 6 or 7 animals each: Group 1, sciatic axonotmesis injury without any other intervention (Group 1-Crush); Group 2, the axonotmesis lesion of 3 mm was infiltrated with a suspension of 1 250 −1 500 human mesenchymal stem cells (total volume of 50 μL) (Group 2-CrushCell); Group 3, axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane covered with a monolayer of non-differentiated human mesenchymal stem cells (Group 3-CrushChitIIICell) and Group 4, axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane (Group 4-CrushChitIII). Motor and sensory functional recovery was evaluated throughout a healing period of 12 weeks using sciatic functional index, static sciatic index, extensor postural thrust, and withdrawal reflex latency. Stereological analysis was carried out on regenerated nerve fibers. Results showed that infiltration of human mesenchymal stem cells, or the combination of chitosan membrane enwrapment and human mesenchymal stem cell enrichment after nerve crush injury provide a slight advantage to post-traumatic nerve regeneration. Results obtained with chitosan type III membrane alone confirmed that they significantly improve post-traumatic axonal regrowth and may represent a very promising clinical tool in peripheral nerve reconstructive surgery. Yet, umbilical cord human mesenchymal stem cells, that can be expanded in culture and induced to

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form several different types of cells, may prove, in future experiments, to be a new source of cells for cell therapy, including targets such as peripheral nerve and muscle.

Key Words

stem cells; mesenchymal stem cells; Wharton jelly; umbilical cord; biomaterials; chitosan; axonotmesis; functional analysis; rat; karyotype analysis; stereological analysis

Research Highlights

(1) The sought for effective new therapeutic strategies for improving peripheral nerve regeneration represents one of the hot topics in biomedicine because of the high number of lesions affecting peripheral nerves.
(2) In this study, it was tested *in vivo* the application of human mesenchymal stem cells from Wharton's jelly of the umbilical cord associated to hybrid chitosan, focusing on its effect in promoting nerve regeneration in axonotmesis.
(3) The results open interesting perspectives in regenerative medicine/tissue engineering emerging areas for the clinical employment of human mesenchymal stem cells and chitosan in peripheral nerve reconstruction.
(4) These results can be interesting for an interdisciplinary readership.

Abbreviations

GPTMS, γ-glycidoxypropyltrimethoxysilane; ESCs, embryonic stem cells; MSCs, mesenchymal stem cells; SFI, sciatic functional index; SSI, static sciatic index; WRL, withdrawal reflex latency

INTRODUCTION

A full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remains the principle goal of regenerative biology. The reliability of animal models is crucial for peripheral nerve research. Because of its peripheral nerve size, the rat sciatic nerve has been the most commonly experimental model used in this kind of studies[1]. The induction of a crush injury in rat sciatic nerve provides a very realistic and useful model of damage for the study of the role of numerous factors in regenerative processes[2]. Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After axonotmesis injury, regeneration is usually successful, after a short (1–2 days) latency, axons regenerate at a steady rate towards the distal nerve stump, supported by the reactive Schwann cells and the preserved endoneural tubules enhance axonal elongation and facilitate adequate reinnervation[3].
Chitosan has attracted particular attention in medical areas due to its biocompatibility, biodegradability, and low toxicity, low cost, enhancement of wound-healing and antibacterial effects[2, 4]. In addition, the potential usefulness of chitosan in nerve regeneration has been demonstrated both *in vitro* and *in vivo*[5-6]. Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin[5-6].

While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups. Finally, the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. By adding GPTMS and employing a freeze-drying technique, we have previously obtained chitosan type III membranes (hybrid chitosan membranes) with pores of about 110 μm diameter and about 90% of porosity, and which were successful in improving sciatic nerve regeneration after axonotmesis[2, 4]. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported and it has been shown that they retain about two times as much biological fluid[2, 4, 7].
Tissue engineering associates biomaterials, like chitosan, to cellular systems, able to differentiate into neuron-like cells, which might improve peripheral nerve regeneration, in terms of motor, sensory and histomorphometric

parameters. Schwann cells, mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), bone marrow stromal cells are the most studied cells candidates. With only a few exceptions, adult stem cell are difficult to expand in culture, and multipotency is a property that remains mostly observed *in vivo*, whereas ESCs show a remarkable capacity to differentiate into a wide range of cell types *in vitro*[8-9]. Most human ESC lines have a normal karyotype. Chromosomal abnormalities are common in embryonic carcinomal cells and mouse ESCs, and karyotypic changes often enhance their proliferative capacity while shortening the population doubling time. Such epigenetic changes are associated with prolonged culture of ESCs[10-11] and are observed not only in naturally occurring ESCs, but also in ESCs cloned using somatic cell nuclear transfer[12]. It has also been reported that the acquisition of chromosomal abnormalities may be related to the laboratory manipulations of cells. Established cell lines must be maintained under stringent culture conditions and be checked often for the acquisition of chromosomal abnormalities, although the incidence of such instability is not fully understood. So we decided to check the karyotype of the Wharton's jelly MSCs used for nerve regeneration associated with the hybrid chitosan membranes type III. The cellular systems implanted into the injured nerve may produce growth factors or extracellular matrix molecules, or may modulate the inflammatory process, to improve nerve regeneration[4, 13-16]. We previously focused our research in N1E-115 cell line differentiated *in vitro* [4, 13, 15-16]. To implant cultured cells (N1E-115 cells, MSCs, Schwann cells, and other cellular systems) into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). It can also be performed by pre-adding the cells to the neural scaffold *via* injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve.

In addition to the ESCs, there are sources rich in non-ESCs, or adult stem cells. These are undifferentiated cells found in differentiated tissues that have limited self-renewal and differentiation capacity, usually restricted to cell types of the tissue that they originally came from. The most common source from which they are isolated is the bone marrow, a mesoderm derived tissue. For decades, it has been known that the bone marrow contains two types of stem cells: hematopoietic ones, which are committed to differentiate into mature blood cells, and the less-differentiated MSCs. MSCs have the ability to differentiate *in vivo* and *in vitro* into a variety of adult mesenchymal tissues, such as bone, cartilage, adipose, and muscle and therefore may be considered to be an important counterpart for potential clinical applications. In addition to the cells derived from the bone marrow, the term "adult stem cell" also describes cells obtained from less-mature sources such as the umbilical cord blood, the placenta, and fetal tissues such as the umbilical cord, the liver and pancreas[17]. Extra-embryonic tissues as stem cell reservoirs offer many advantages over both embryonic and adult stem cell sources. Extra-embryonic tissues, collectively known as the afterbirth, are routinely discarded at parturition, so little ethical controversy attends the harvest of the resident stem cell populations. Most significantly, the comparatively large volume of extra-embryonic tissues and easy manipulation hypothetically increases the number of stem cells that can be isolated[18]. The umbilical cord contains two arteries and one vein protected by a proteoglycan rich connective tissue called Wharton's jelly. Within the abundant extracellular matrix of Wharton's jelly resides a recently described stem cell population called umbilical cord matrix stem cells or Wharton's jelly MSCs. In average, 400 000 cells can be isolated per umbilical cord, which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow. The phenotypic stromal cells in the Wharton's jelly are fibroblast-like cells[17]. However, cells with the ultra-structural characteristics of myofibroblasts have been found[17]. The MSCs from the umbilical cord express adhesion molecules (CD44, CD105), integrin markers (CD29, CD51), and MSC markers (SH2, SH3) but not markers of hematopoietic differentiation (CD34, CD45)[17], when expanded in culture. *In vitro*, Wharton's jelly MSCs are capable of differentiation to multiple mesoderm cell types including skeletal muscle and neurons[17-19]. Generation of clinically important dopaminergic neurons has also been reported[19]. Human MSCs isolated from Wharton's jelly from the umbilical cord can be easily and ethically obtained and processed compared with embryonic or bone marrow stem cells. These cells may be a valuable source in the repair of the peripheral nerve system. Human MSCs from Wharton's jelly of the umbilical cord possess stem cell properties[20] and it was previously demonstrated that human MSCs could be induced to differentiate into neuron-like cells[19]. The transplanted cells were able to promote local blood vessel formation (local vascularization) and produce neurotrophic factors, like brain-derived neurotrophic

factor and glial cell line-derived neurotrophic factor[17, 19]. Thus, umbilical cord human MSCs can be expanded in culture and induced to form several different types of cells. They may therefore prove to be a new source of cells for cell therapy, including targets such as peripheral nerve and muscle. This will help to avoid several ethical and technical issues. Previous published results from *in vivo* experiments showed that enrichment of chitosan membranes with N1E-115 neural cells in axonotmesis and neurotmesis lesions did not have any positive effect on nerve regeneration in comparison to crush controls and, in case of type III chitosan membrane, the presence of transplanted cells even prevented the positive effects of the membrane wrapping alone on nerve regeneration. Probably, these negative results obtained were due to the neoplastic source of the cellular system used for neurotrophic factors delivery albeit the *in vitro* differentiation into neural cells in the presence of DMSO[2, 13, 16, 21]. Neuronal differentiation of these cells is accompanied by synthesis and delivery of a number of neurotrophic factors that might be useful in promoting axonal elongation[2, 13, 16, 21]. However, the presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration[2, 13, 16, 21]. Anyway, it can be hypothesized that membrane enrichment with other cell types like the MSCs, may lead to better results and thus the goal of this study was to assess the effect of the hybrid chitosan membrane type III enriched with non-differentiated human MSCs isolated from umbilical cord Wharton's jelly, in peripheral nerve reconstruction after crush injury. The synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III compared to common chitosan, as well as the presence of silica ions, may be responsible for the good results in promoting post-traumatic nerve regeneration[2] suggesting that this material may not just work as a simple mechanical scaffold but instead may work as an inducer of nerve regeneration[2]. The neuroregenerative property of chitosan type III might be explained by a direct stimulation of Schwann cell proliferation, axon elongation and myelination[22-23].

RESULTS

Human MSCs culture and karyotype analysis

Undifferentiated human MSCs from human umbilical cord Wharton's jelly, exhibited a normal star-like shape with a flat morphology in culture (Figure 1). A total of 20 Giemsa-stained metaphases of these cells, were analyzed for numerical aberrations. Sporadic, non-clonal aneuploidy was found in 3 cells (41–45 chromosomes). The other 17 metaphases had 46 chromosomes (Figure 2). The karyotype was determined in a completely analyzed G-banding metaphase. No structural alterations were found. The karyotype analysis to the human MSCs cell line derived from human Wharton jelly demonstrated that this cell line has no neoplastic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes.

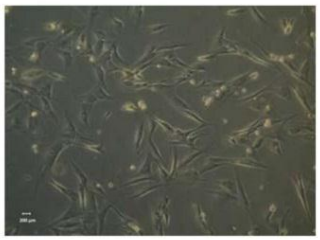

Figure 1 Undifferentiated mesenchymal stem cells, from human umbilical cord Wharton's jelly, exhibiting a star-like shape with a flat morphology (× 100).

Figure 2 Selected methaphases from human mesenchymal stem cell line from Wharton's jelly, showing the normal number of chromosomes (46, XY) (× 1 000).

Functional analysis of motor deficit and nociceptive function

The withdrawal reflex latency (WRL) test was used to assess nociception. In the first 2 weeks post sciatic crush injury, the WRL response was absent (*i.e.* lack of withdrawal within 12 seconds; the cutoff time to prevent thermal damage to the paw) in a large majority of animals (Table 1). With time, the WRL improved in all animals and at week 12, the WRL values were 2.00 ± 0.00, 1.29 ± 0.18, 1.17 ± 0.17, and 3.3 ± 0.54 seconds, in

groups Crush, CrushCell, CrushCellChitIII, and CrushChitIII, respectively. A significant difference was observed between the groups in WRL data ($F_{(3,22)}$ = 6.449, P = 0.001) with delayed recovery in WRL performance in the CrushChitIII group compared to the other three groups (P < 0.05).

The sciatic nerve crush caused severe muscle force deficit in the affected limb immediately post-surgery. At week 1, the percentage of motor function deficit for the right hindlimb reached over 90% in all groups (Table 2). A gradual recovery of the right hindlimb extensor postural thrust occurred during the 12-week survival time in all groups, so that at week 12, the EPT deficit in the affected side, although not fully reestablished, was reduced to only 4.16 ± 5.60, 2.79 ± 0.41, 3.24 ± 0.42, and 7.43 ± 3.58 in groups Crush, CrushCell, CrushChitIIICell and, CrushChitIII, respectively. EPT performance was similar in all groups [$F_{(3,22)}$ = 1.367, P = 0.279] (Table 2) although at the end of the 12 weeks of recovery, the EPT values were lower in CrushCell and CrushChitIIICell groups.

Sciatic nerve morphology and stereology

Figure 3 represents the histology of the sciatic regenerating nerves 12 weeks after crush lesion-Group Crush, crush lesion with human MSCs injection-Group CrushCell, crush lesion enwrapped with a chitosan type III membrane covered with human MSCs-Group CrushChitIIICell, crush lesion enwrapped with a chitosan type III membrane-Group CrushChitIII compared to a normal sciatic nerve. Fiber regeneration was good in all experimental groups, though the regenerated nerves presented smaller myelin fibers than the normal nerves without injury, as confirmed by stereological data presented in Table 3.

Table 1 Nociceptive function of rats throughout a healing period of 12 weeks

Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 10	Week 12
Crush											
1A	2	3	3	3	2	2	2	2	2	2	2
1B	1	12	10	5	5	2	3	3	4	3	2
1C	2	10	2	2	2	3	2	2	2	2	2
1D	2	12	10	5	5	3	2	2	2	2	2
1E	1	4	4	3	3	2	2	2	2	2	2
1F	2	12	12	11	10	4	3	3	2	2	2
Mean±SD	1.67±0.52	8.83±4.22	6.83±4.31	4.83±3.25	4.50±3.02	2.67±0.82	2.33±0.52	2.33±0.52	2.33±0.82	2.17±0.41	2.00±0.00
CrushCell											
2A	2	9	8	6	4	3	3	3	2	2	1
2B	1	12	11	8	5	3	3	2	2	1	1
2C	2	12	12	8	5	3	2	2	2	1	1
2D	2	11	7	7	4	3	3	2	1	1	1
2E	1	8	7	6	4	3	3	2	2	2	1
2F	1	12	12	11	10	5	2	2	2	2	2
2G	1	12	8	6	6	4	2	2	2	2	2
Mean±SD	1.43±0.53	10.86±1.68	9.29±2.29	7.43±1.81	5.43±2.15	3.43±0.79	2.57±0.53	2.14±0.38	1.86±0.38	1.57±0.53	1.29±0.49
CrushChitIIICell											
4A	1	10	8	8	7	4	4	2	2	1	1
4B	2	12	8	6	5	4	4	3	2	2	2
4C	1	8	8	8	7	5	5	3	2	1	1
4D	2	11	9	6	4	4	4	3	2	1	1
4E	1	12	8	7	3	3	3	3	1	1	1
4F	2	12	12	8	5	2	2	2	2	1	1
Mean±SD	1.50±0.55	10.83±1.60	8.83±1.60	7.17±0.98	5.17±1.60	3.67±1.03	3.67±1.03	2.67±0.52	1.83±0.41	1.17±0.41	1.17±0.41
CrushChitIII											
3A	4	8	12	9	7	6	6	5	5	4	3
3B	4	6	7	6	6	5	5	4	4	4	3
3C	3	8	12	10	10	6	5	4	3	3	4
3D	2	7	12	10	7	6	4	5	4	3	3
3E	2	12	12	9	8	6	6	5	4	4	3
3F	3	5	11	6	5	5	5	4	4	3	3
3G	3	12	12	9	8	8	7	6	5	5	3
Mean±SD	3.22±0.87	8.11±2.72	10.93±1.96	8.65±1.81	7.15±1.51	5.95±1.02	5.46±1.04	4.89±0.77	4.27±0.68	3.78±0.64	3.30±0.54

Values in seconds (s) were obtained in performing WRL test to evaluate the nociceptive function. This test has been performed pre-operatively (week 0), and every week after the surgical procedure until week 12. There were six or seven rats in each experimental group. Group 1, Sciatic crush injury without any other intervention (Crush), Group 2, the crushed sciatic nerve was infiltrated with a suspension of 1 250–1 500 human MSCs (CrushCell); Group 3, the axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane covered with a monolayer of non-differentiated human MSCs (CrushChitIIICell) and Group 4, the axonotmesis lesion of 3 mm was enwrapped with a chitosan type III (CrushChitIII). Each rat within the experimental group was identified with the group number (1–4) and a letter (A–G).

Myelinated fiber density and total number were significantly ($P < 0.05$) higher than in controls in all nerve regeneration groups except for the CrushChitIII group. Axon and fiber diameter and myelin thickness were significantly ($P < 0.05$) lower in the 3 experimental groups compared to control group (normal sciatic nerve without injury). Analysis of the inter-group variability among regenerated groups, showed that CrushChitIII group had significantly ($P < 0.05$) lower fiber density and fiber total number and a higher myelin thickness while no statistically significant differences ($P > 0.05$) were detectable for the remaining histomorphometrical predictors of nerve regeneration (fiber diameter and axon diameter).

Table 3 Histomorphometrical assessment of normal (control) and regenerated sciatic nerves submitted to a standardized sciatic nerve crush injury with non-serrated clamp (week-12 posttraumatic)

Group	Fiber density (*n*/mm^2)	Fiber number (*n*)	Fiber diameter (μm)	Axon diameter (μm)	Myelin thickness (μm)
Crush	20 109±1 232	10 644±423	4.99±0.19	3.48±0.10	0.76±0.05
CrushCell	20 200±4 971	9 806±2 695	5.31±0.69	3.74±0.49	0.78±0.10
CrushChitIIICell	21 514±6 308	11 413±3 752	4.90±0.97	3.41±0.72	0.75±0.14
CrushChitIII	15 533±7 713	7 982±3 092	5.29±1.05	3.50±0.55	1.02±0.22
Control	15 905±287	7 666±190	6.66±0.12	4.26±0.07	1.19±0.03

Results are presented as mean and standard deviation (SD). Group 1 sciatic crush injury without any other intervention (Crush); Group 2; the crushed sciatic nerve was infiltrated with a suspension of 1 250–1 500 human MSCs (CrushCell); Group 3, the axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane covered with a monolayer of non-differentiated human MSCs; (CrushChitIIICell); Group 3, the axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane covered with a monolayer of non-differentiated human MSCs (CrushChitIIICell) and Group 4, the axonotmesis lesion of 3 mm was enwrapped with a chitosan type III (CrushChitIII).

DISCUSSION

Results from *in vivo* testing previously performed by our research group[2] showed that type III chitosan improved nerve fiber regeneration in comparison to control crushed sciatic nerves. Chitosan type III was developed as a hybrid of chitosan by the addition of GPTMS. Wettability of material surfaces is one of the key factors for protein adsorption, cell attachment and migration[24]. The addition of GPTMS improved the wettability of chitosan surfaces[2, 4, 22], and therefore chitosan type III is expected to be more hydrophilic than the original chitosan[2, 4, 22]. Chitosan type III was developed to be more porous, with a larger surface to volume ratio but preserving mechanical strength and the ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported as a consequence of the difference in the ability of the matrix to hold water. In fact, hybrid chitosan-based membranes may retain about twice as much biological fluid as chitosan[7]. A synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III and the presence of silica ions may be responsible for the good results in promoting post-traumatic nerve regeneration. The significant improvement of axonal regeneration obtained in crushed sciatic nerves surrounded by chitosan type III membranes suggests that this material may not just work as a simple mechanical scaffold but instead may work as an inducer of nerve regeneration. The neuroregenerative property of chitosan type III might be explained by the action on Schwann cell proliferation, axon elongation and myelinization[2, 4, 22]. Yet, the expression of established myelin genes such as *PMP22*, *PO* and *MBP*[25-26] may be influenced by the presence of silica ions which exert an effect on several glycoprotein expression[25-26].

Results from *in vivo* experiments previously performed[2] showed that enrichment of chitosan membranes with N1E-115 neural cells did not have any positive effect on nerve regeneration in comparison to crush controls and, in case of type III chitosan membrane, the presence of transplanted cells even prevented the positive effects of the membrane wrapping alone on nerve regeneration. These results are in agreement with previous experiments that showed that N1E-115 cell population does not have significant effects in promoting axon regeneration and, when N1E-115 cells were cultured inside a poly(lactic-co-glycolic acid) scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration[16, 21]. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration[2, 13, 16, 21]. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after

axonotmesis and neurotmesis[2,16, 21] and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplastic origin.

In this study, we used chitosan type III membrane to deliver human MSCs from the umbilical cord Wharton jelly and we compared this delivery approach with direct injection/infiltration of these human MSCs in suspension, in the rat sciatic nerve axonotmesis model. The cellular systems implanted into the injured nerve may produce growth factors or extracellular matrix molecules, or may modulate the inflammatory process, to improve nerve regeneration or even replace the injured neural and Schwann cells[4, 13-16, 27]. The human MSCs karyotype was studied in order to be sure that these cells did not present any number or structure chromosome abnormalities due to isolation and cell culture procedures before *in vivo* application. This concern was due to the negative effects that N1E-115 cells presented in axonotmesis and neurotmesis injuries, since this cell line has neoplastic characteristics[2, 13, 16, 21]. The karyotype analysis to the human MSCs cell line derived from umbilical cord Wharton jelly demonstrated that this cell line has no neoplastic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes. Also, the morphologic characteristics of these cells in culture, observed in an inverted microscope, were perfectly normal. These cells presented a star-like shape with a flat morphology, characteristic of MSCs[28].

The functional analysis revealed a gradual recovery of the injured hindlimb EPT during the 12-week healing period in all three experimental groups. In week 12, the percentage motor deficit in the affected hindlimb, although not totally recovered to normal values, reduced especially in CrushCell, and CrushCellChitIII groups. With time, the WRL improved in all animals during the 12-week healing period, and no differences between groups were found in the rate of recovery of this response. By the end of the 12 weeks, all animals from the three experimental groups presented normal WRL values. Anyway, there was no significant delayed recovery in WRL performance in the CrushChitIII group compared to the other three groups.

Similarly, stereological analysis showed no statistically significant differences among the experimental groups for any of the histomorphological of nerve regeneration investigated with the only exception of the group where nerve crush site was enwrapped with chitosan type III membranes alone, in line with previous findings[2]. The neuroregenerative property of chitosan type III might be explained by the action on Schwann cell proliferation, axon elongation and myelinization[2, 4, 22], which might explain the higher myelin thickness in the regenerated nerves enwrapped with the chitosan type III membrane alone (Group CrushChitIII). Comparing the results obtained in crush injuries where the local lesion was enwrapped with chitosan type III membranes associated to N1E-115 *in vitro* differentiated cells[2], with the results obtained in this experimental work with the same chitosan membranes associated to human MSCs, we conclude that the negative effects observed with the N1E-115 cell line are not observed with human MSCs. As a matter of fact, the application of human MSCs associated or not to the chitosan membranes showed positive effects concerning the functional recovery (evaluated by EPT and WRL tests), probably due to the modulation of the inflammatory process during the Wallerian degeneration and by the production of growth factors. On the other hand, statistically significant positive effects where observed concerning the higher myelin thickness in the regenerated nerves enwrapped with the chitosan type III membrane alone. As expected, regenerated nerve fibers were organized in microfascicles and smaller when compared to normal control nerves. CrushChitIII group like the control group presented a significantly lower fiber density and fiber total number and a higher myelin thickness in comparison with other experimental groups as it was observed in a previous published work[2].

In conclusion, results of this study suggest that either enrichment of human MSCs alone or the combination of chitosan type III membrane enwrapment and human MSCs infiltration after nerve crush injury provides a slight advantage in comparison to untreated controls. On the other hand, our results confirmed that chitosan type III membranes alone may represent a very promising clinical tool in peripheral nerve reconstructive surgery. Thus, human umbilical cord human MSCs can be expanded in culture and induced to form several different types of cells. They may therefore in future experiments, be tested as a new source of cells for cell therapy, including targets such as peripheral nerve in more serious lesions (neurotmesis with and without loss of nerve tissue) and muscle. Also, a more accurate functional analysis in other to evaluate different therapeutic strategies should be used, considering for instance the use of ankle kinematic analysis during the rat locomotion.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This experimental work was performed from January to July of 2011 in Institute of Biomedical Sciences Abel Salazar, Veterinary Clinics Department, Porto University, Portugal.

Materials

Hybrid chitosan membranes

Chitosan (high molecular weight, Aldrich®, USA) was dissolved in 0.25 M acetic acid aqueous solution to a concentration of 2% (w/v). To obtain type III membranes, GPTMS (Aldrich®, USA) was also added to the chitosan solution and stirred at room temperature for 1 hour. The drying process for type III chitosan membrane was as follows: the solutions were frozen for 24 hours at –20°C and then transferred to the freeze-dryer, where they were left 12 hours to complete dryness. The chitosan type III membranes were soaked in 0.25 N sodium hydroxide aqueous solution to neutralize remaining acetic acid, washed well with distilled water, and freeze dried[2]. All membranes were sterilized with the preferred ethylene oxide gas method[29]. Prior to their use *in vivo*, membranes were kept during 1 week at room temperature in order to clear any ethylene oxide gas remnants[2].

Methods

Human MSCs culture

Human MSCs from Wharton's jelly umbilical cord matrix were purchased from PromoCell GmbH (C-12971, lot No. 8082606.7). The human MSCs were cultured and maintained in a humidified atmosphere with 5% CO_2 at 37°C. MSC medium, PromoCell (C-28010) was replaced every 48 hours. At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco, Alfagene, Carcavelos, Portugal) and passed into a new flask for further expansion. MSCs at a concentration of 10^4 cells/cm^2 were cultured exhibiting a 90% confluence after 4 days. The application of human MSCs in rats is possible without inducing any immunossupression in the experimental animals.

Karyotype determination of human MSCs from Wharton's jelly

Chromosome analysis on human MSC line from Wharton's jelly was carried out between passages 4 and 5. When confluence was reached, culture medium was changed and supplemented with 4 μg/mL colcemid solution (stock solution, Cat. nº. 15212-012, Gibco). After 4 hours, cells were collected and suspended in 8 mL of 0.075 M KCl solution supplemented with bovine fetal serum. Then the suspension was incubated in 37°C for 35 minutes. After centrifugation (1 500 r/min), 8 mL of the fixative methanol: glacial acetic acid at 6:1 was added and mixed together, and the cells were again centrifuged. After two rounds of fixation, two new rounds were performed with the fixative methanol: glacial acetic acid at 3:1. After the last centrifugation, the cell suspension was spread onto very well cleaned slides. Chromosome analysis was performed by one scorer on 20 Giemsa-stained metaphases. Each cell was scored for chromosome number. Routine chromosome G-banding analysis was also carried out for determination of the karyotype.

Surgical procedure

All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC). A total of 25 adult male Sasco Sprague rats (Charles River Laboratories, Barcelona, Spain) weighing approximately 250 g at the start of the experiment were used. Animals were divided by 4 experimental groups of 6 or 7 animals each. Experimental groups were set according to treatment after nerve sciatic axonotmesis injury. In Group 1, animals recovered from axonotmesis sciatic injury without any other intervention (Group 1–Crush). In Group 2, axonotmesis sciatic nerve was infiltrated with a suspension of 1 250–1 500 MSCs (total volume of 50 μL) (Group 2–CrushCell). In Group 3, axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane covered with a monolayer of non-differentiated human MSCs (Group 3–CrushChitIIICell) and in Group 4, axonotmesis lesion of 3 mm was enwrapped with a chitosan type III membrane (Group 4–CrushChitIII). Standardized crush injury was carried out with the animals placed prone under sterile conditions and skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. Surgery procedure was previously described[3, 15]. A standard crush injury was performed by a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30 seconds, 10 mm above the bifurcation into tibial and common peroneal nerves, inducing a 3 mm axonotmesis lesion[30]. To prevent autotomy, a deterrent substance was daily applied to rat right foot[31]. Animals were intensively examined for signs of autotomy and contracture and none presented severe wounds (absence of a part of the foot or severe infection) or contractures during the study. No local or systemic signs of rejection or foreign body were observed in the experimental animals transplanted with chitosan type III membranes and human MSCs. There was no need of

administrating immunosuppressive treatment to the experimental animals during the entire healing period of 12 weeks after the surgical procedure.

Functional analysis of motor deficit and nociceptive function

All animals were tested preoperatively (week 0), every week until week 8, and then every other week, until week 12. Animals were gently handled, and tested in a quiet environment to minimize stress levels. Motor deficit and nociceptive function were evaluated by measuring EPT and WRL, respectively[3, 15-16, 21]. For EPT test, the entire body of the rat, excepting the hindlimbs, was wrapped in a surgical towel. Supporting the animal by the thorax and lowering the affected hind limb towards the platform of a digital balance, the EPT was elicited. As the animal was lowered to the platform, it extended the hindlimb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance (model TM 560; Gibertini, Milan, Italy) was recorded. The same procedure was applied to the contra-lateral, unaffected limb. For this test, the affected and normal limbs were tested three times, with an interval of 2 minutes between consecutive tests, and the three values were averaged to obtain a final result. Normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into an equation (Equation (1)) to derive the percentage of functional deficit, as described in the literature[32]:

$$\%\text{Motor deficit} = [(\text{NEPT} - \text{EEPT}) / \text{NEPT}] \times 100$$

Nociceptive WRL was adapted from hotplate test developed by Masters *et al* [33] and described elsewhere[3, 15-16, 21]. Briefly, the rat was wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate at 56°C (model 35-D, IITC Life Science Instruments, Woodland Hill, CA, USA). WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. Normal rats withdraw their paws from the hotplate within 4 seconds or less[34]. The affected limbs were tested 3 times, with an interval of 2 minutes between consecutive tests to prevent sensitization, and the 3 latencies were averaged to obtain a final result. The cutoff time for heat stimulation was set at 12 seconds to avoid skin damage to the foot[35].

Sciatic nerve morphology and stereology

Nerve samples (10-mm-long sciatic nerve segments distal to the crush site and from un-operated controls) were processed for quantitative morphometry of myelinated nerve fibers[36]. Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1 M Sorensen phosphate buffer for 6–8 hours and resin embedding was obtained following Glauerts' procedure[37]. Series of 2 µm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology was carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector were adopted using a protocol previously described[38-39]. Fiber density and total number were estimated together with fiber and axon diameter and myelin thickness.

Statistical analysis

The results of the functional tests are reported for each time point, including pre-operatively, and each experimental group as means and standard deviation (SD). Differences between time points and between groups were tested by two-way analysis of variance using a mixed model of within- (time of recovery) and between-subjects (experimental groups) factors. Pairwise comparisons between each two groups were undertaken using the post hoc Tukey's HSD test. Statistical significance was accepted at the level of $P < 0.05$. For stereology, statistical comparisons of quantitative data were subjected to one-way analysis of variance test. Statistical significance was established as $P < 0.05$. All statistical procedures were performed by using the statistical package SPSS (version 14.0, SPSS, Chicago, IL, USA) except stereological data that were analyzed using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milan, Italy).

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rats' daily treatment, Paulo AS Armada-da-Silva performed the statistical analysis, Rosa Sousa and Beatriz Porto performed the karyotype analysis, Simone Bompasso and S Raimondo performed the histomorphometric analysis, Yuki Shirosaki, Yuri Kakamura, Satoshi Hayakawa and Akiyoshi Osakah were responsible for the chitosan membranes development and production, Ana Lúcia Luís, Artur SP Varejão, Miguel L França, Tiago Pereira and Ana Colette Maurício performed animal surgeries, Andrea Gärtner, Tiago Pereira and Ana Colette Maurício designed the experimental protocol, Paulo AS Armada-da-Silva, Andrea Gärtner, Tiago Pereira, Artur SP Varejão, Ana Lúcia Luís and Ana Colette Maurício wrote the manuscript and prepared the tables and figures.

Conflicts of interest: None declared.

Ethical approval: In this study, all procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed.

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1.3

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Use of poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly of the umbilical cord for promoting nerve regeneration in axonotmesis: *In vitro* and *in vivo* analysis

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ABSTRACT

Cellular systems implanted into an injured nerve may produce growth factors or extracellular matrix molecules, modulate the inflammatory process and eventually improve nerve regeneration. In the present study, we evaluated the therapeutic value of human umbilical cord matrix MSCs (HMSCs) on rat sciatic nerve after axonotmesis injury associated to Vivosorb® membrane. During HMSCs expansion and differentiation in neuroglial-like cells, the culture medium was collected at 48, 72 and 96 h for nuclear magnetic resonance (NMR) analysis in order to evaluate the metabolic profile. To correlate the HMSCs ability to differentiate and survival capacity in the presence of the Vivosorb® membrane, the $[Ca^{2+}]i$ of undifferentiated HMSCs or neuroglial-differentiated HMSCs was determined by the epifluorescence technique using the Fura-2AM probe. The Vivosorb® membrane proved to be adequate and used as scaffold associated with undifferentiated HMSCs or neuroglial-differentiated HMSCs. *In vivo* testing was carried out in adult rats where a sciatic nerve axonotmesis injury was treated with undifferentiated HMSCs or neuroglial differentiated HMSCs with or without the Vivosorb® membrane. Motor and sensory functional recovery was evaluated throughout a healing period of 12 weeks using sciatic functional index (SFI), extensor postural thrust (EPT), and withdrawal reflex latency (WRL).

Stereological analysis was carried out on regenerated nerve fibers. *In vitro* investigation showed the formation of typical neuroglial cells after differentiation, which were positively stained for the typical specific neuroglial markers such as the GFAP, the GAP-43 and NeuN.

NMR showed clear evidence that HMSCs expansion is glycolysis-dependent but their differentiation requires the switch of the metabolic profile to oxidative metabolism. *In vivo* studies showed enhanced recovery of motor and sensory function in animals treated with transplanted undifferentiated and differentiated HMSCs that was accompanied by an increase in myelin sheath. Taken together, HMSC from the umbilical cord Wharton jelly might be useful for improving the clinical outcome after peripheral nerve lesion.

1. Introduction

A full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remain the principle goal of regenerative medicine. The reliability of animal models is crucial for peripheral nerve research. Because of its peripheral nerve size, the rat sciatic nerve has been the most commonly experimental model used in studies concerning the peripheral nerve regeneration and possible therapeutic approaches (Ronchi et al., 2009). Although sciatic nerve

Abbreviations: NMR, nuclear magnetic resonance; SFI, sciatic functional index; EPT, extensor postural thrust; WRL, withdrawal reflex latency; PNS, peripheral nervous system; PLC, poly(DL-lactide-ε-caprolactone); PLGA, poly(lactic-co-glycolic acid)

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injuries are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Amado et al., 2008; Mackinnon et al., 1985). Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After this kind of injury, regeneration is usually successful, in a short latency (1–2 days), axons regenerate at a steady rate along the distal nerve supported by the reactive Schwann cells (SCs) and the preserved endoneural tubules which enhance axonal elongation and facilitate adequate reinnervation (Luis et al., 2007).

Tissue engineering of peripheral nerves associates biomaterials, like chitosan, PLC, and other biomaterials, some of them, previously studied by our group (Amado et al., 2008; Luis et al., 2008a; Maurício et al., 2011) to cellular systems, able to differentiate into neuroglial like cells or even by modulating the inflammatory process, which might improve nerve regeneration, in terms of motor and sensory recovery, and also, by shortening the healing period avoiding regional muscular atrophy. Cell transplantation has been proposed as a method of improving peripheral nerve regeneration (Chen et al., 2007). SCs, MSCs, embryonic stem cells, marrow stromal cells are the most studied support cells candidates. Schwann cell transplantation enhances axon outgrowth both *in vitro* (Schlosshauer et al., 2003) and *in vivo* (Keilhoff et al., 2006). Transplantation of viable SCs offers better results than the sheer release of growth factors, because SCs have the above mentioned regeneration promoting effect (Bhatheja and Field, 2006; Fansa et al., 1999; Hall, 1978; Ide, 1996; Madduri and Gander, 2010; Muir, 2010; Schmitte et al., 2010). The generation of sufficient amounts of SCs for auto-transplantation, however, requires a significant time for cell culture. Current concepts include mitogenic substances to enhance cell yield. Due to their side effects they should be avoided in clinical therapy. Moreover, functional nerves have to be sacrificed as SCs donor resulting in loss of sensation, scarring and, possibly, neuroma formation. The limitations of harvesting autologous SCs, such as donor nerve sacrifice and donor site morbidity, have led to investigation of other cell types that provide similar trophic support to axon regeneration (Ladak et al., 2011).

On the other hand, MSCs have become one of the most interesting targets for Tissue Regeneration including the peripheral nerves. Like bone marrow stromal cells and other mesenchymal cells, they are plastic adherent, stain positively for markers of the mesenchymal (CD10, CD13, CD29, CD44, CD90, and CD105) and negatively for markers of the hematopoietic lineage (Wang et al., 2004). These cells are capable of self-renewal with sustained proliferation *in vitro* and can differentiate into multiple mesodermal cells, including neuron-like cells (Park et al., 2010). The high plasticity and low immunogenicity of these cells, turn them into a desirable form of cell therapy for the injured nervous system without requiring the use of immunosuppressive drugs during the treatments (Thuret et al., 2006). MSCs have been isolated from various other origins, including skin, hair follicle, periosteum, amniotic fluid, umbilical cord blood and adipose tissue. Harvest and expansion of MSCs are straight forward techniques (Colter, 2000; Gnecchi and Melo, 2009), which are not only feasible regarding the laboratory approach but also regarding their application with the patient. With appropriate stimuli and environmental conditions MSCs exhibit a respectable plasticity (Joshi and Enver, 2002). They may even differentiate into non-mesenchymal lineages, including myelinating cells of the peripheral nervous system (PNS) (Dezawa et al., 2001; Tohill et al., 2004). Beside their easy expansion in culture their plasticity makes MSCs an ideal source for tissue repair and tissue engineering. Bone marrow represents the most commonly used tissue source of adult MSCs. Bone marrow MSCs (BMSCs) have been applied for cell based therapies; however, due to the limited number of BMSCs available for autologous use and the possibility of donor site morbidity and the decreased number of BMSCs along the adult life, there is a need to identify alternative MSCs sources.

A recently reported potential alternative tissue source of MSCs is the connective tissue (Wharton's Jelly) of human umbilical cord (UC). Different harvesting procedures have led to UC-derived cells that exhibit a neuronal phenotype (Fu et al., 2006; Mitchell et al., 2003; Sarugaser et al., 2005; Wang et al., 2004) and have potential utility in treatment of neurodegenerative diseases (Weiss et al., 2003, 2006), indicating the versatility of this cell source. Interestingly, these cells, which are major histocompatibility complex (MHC) class II negative, not only express immunoprivileged and immunomodulatory phenotype, but also their MHC class I expression levels can be manipulated (Sarugaser et al., 2005), making them a potential cell source for MSC-based therapies. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses (Maurício et al., 2011).

The aim of this study was to explore the therapeutic value of human UC matrix (Wharton's jelly) derived MSCs (HMSC) both *in vitro* and *in vivo*, associated to a Poly(DL-lactide-ε-caprolactone) PLC (Vivosorb®) membrane, on a rat sciatic nerve axonotmesis experimental model.

2. Materials and methods

2.1. Poly(DL-lactide-ε-caprolactone) (PLC) membranes

Poly(DL-lactide-ε-caprolactone) (PLC) membranes (Vivosorb®) were purchased from Polyganics BV, Groningen, Netherlands (FS01-006/20 Lot: FSA2009092311).

2.2. Cell culture and in vitro differentiation of HMSC from Wharton's jelly umbilical cord

Human MSC from Wharton's jelly UC (HMSCs) were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreservated cells were cultured and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 h. At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. HMSCs at a concentration of 2500 cell/ml were cultured and after 24 h cells exhibited 30–40% confluence. Differentiation was induced with MSC neurogenic medium (Promocell, C-28015). Medium was replaced every 24 h for 3 days. The formation of neuroglial-like cells was observed after 24 h in an inverted microscope (Zeiss, Germany).

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) was measured in Fura-2-loaded cells by using dual wavelength spectrofluorometry as previously described (Amado et al., 2008). The measurements were performed on undifferentiated HMSCs after confluence was obtained and on neuroglial-differentiated HMSCs, cultured on Vivosorb® discs in order to correlate the HMSCs ability to differentiate and survival capacity in the presence of the Vivosorb® membrane.

2.3. Cytogenetic analysis of human MSC from Wharton's jelly

HMSC cell line (differentiated) from Wharton's jelly was studied for cytogenetic analysis at passage 5. When confluence was reached, culture medium was changed and supplemented with 4 μg/ml colcemid solution (stock solution, Cat. No. 15212-012, Gibco). After 4 h, HMSCs were collected and suspended in

8 ml of 0.075 M KCl solution supplemented with bovine fetal serum (BFS). Then the suspension was incubated at 37 °C for 35 min. After centrifugation (1500 rpm), 8 ml of the fixative methanol: glacial acetic acid at 6:1 was added and mixed together, and the cells were again centrifuged. Aftertwo rounds of fixation, two new rounds were performed with the fixative methanol: glacial acetic acid at 3:1. After the last centrifugation, the HMSC suspension was spread onto very well glass cleaned slides. Analysis was performed by one scorer on Giemsa-stained cells.

2.4. Immunocytochemistry

At passage 3, HMSCs were trypsinized, washed and re-suspended in Mesenchymal Stem Cell Medium (PromoCell, C-28010) at a concentration of 1×10^5 cell/ml. HMSCs were fixed with paraformaldehyde at 4 °C for 15 min and washed with distilled water before permeabilization in 0.5% Triton-X100. Non-specific binding was blocked using blocking solution (PBS containing 1% bovine serum albumin (BSA)) for 1 h at room temperature. HMSCs were then incubated for 2 h at room temperature with primary antibodies of rabbit anti-growth associated protein-43 (GAP-43, 1:200) (Chemicon, AB5220), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500) (Chemicon, AB5804) and mouse anti-neuronal nuclei (NeuN, 1:100) (Chemicon, MAB377). After washing, HMSCs were incubated 15 min with secondary antibodies goat anti-rat IgG (Millipore, AP136P) and goat anti-rabbit IgG (Millipore, 12-348MN). After several washes in PBS, HMSCs were incubated with horseradish peroxidase (HRP)-coupled streptavidin for 10 min. DAB (diaminobenzidine) served as chromogen.

2.5. NMR spectroscopy

During HMSCs expansion and differentiation to neuroglial-like cells, 160 μL of the culture medium was collected at 48, 72 and 96 h ($N=5$) for nuclear magnetic resonance (NMR) analysis. 1 H-NMR spectra of the collected samples were acquired at 14.1 *T*, 25 °C, using a Varian 600 MHz spectrometer equipped with a 3 mm indirect detection probe with z-gradient (Varian, Palo Alto, CA) by standard methods. Solvent-suppressed 1 H-NMR spectra were acquired with 6 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3 s water pre-saturation, 45° pulse angle, 3.5 s acquisition time, and at least 64 scans. 1H-NMR spectroscopy was performed and the following metabolites were determined: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm; and H1-α glucose, doublet at 5.22 ppm. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution. The relative areas of 1 H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

2.6. Surgical procedure

For the *in vivo* testing, Sasco Sprague adult rats (Charles River Laboratories, Barcelona, Spain) were divided in groups of 6 animals each: a group of 6 animals was used as control without any sciatic nerve injury (Group 1 – Control). In Group 2 the crushed sciatic nerve did not have any other intervention (Group 2 – Crush). In Group 3, the axonotmesis lesion of 3 mm was enwrapped with a PLC (Vivosorb®) membrane (Group 3 – CrushPLC). In Group 4, the crushed sciatic nerve was infiltrated in the lesion area with a suspension of 1500 HMSCs (in a total volume of 50 μl) (Group 4 – CrushCell), in Group 5, the crushed sciatic nerve was encircled by a PLC (Vivosorb®) membrane covered with a monolayer of non (Group 5 – *CrushCellNonDifPLC*) and in Group 6 the axonotmesis lesion of 3 mm was enwrapped with a PLC (Vivosorb®) membrane covered with a monolayer of differentiated HMSCs (neuroglial-like cells) (Group 6 – CrushCell-DifPLC). The standardized crush injury was carried out with the animals placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. The surgery procedure was the one previously described (Luís et al., 2007; Luis et al., 2007). The standard crush injury was performed by a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3 mm axonotmesis lesion. To prevent autotomy, a deterrent substance was applied to rat right foot. No local or systemic signs of rejection or foreign body were observed in the experimental animals transplanted with PLC membranes and HMSCs. There was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period of 12 weeks after the surgical procedure.

2.7. Functional assessment

All animals were tested preoperatively (week 0), and every week until the end of the 12-week follow-up time.

2.7.1. Motor performance and nociceptive function

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively (Luis et al., 2007, 2008a, b). For EPT test, the affected and normal limbs were tested 3 times, with an interval of 2 min between consecutive tests, and the 3 values were averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into an equation (Eq. (1)) to derive the percentage of functional deficit, as described in the literature(Koka and Hadlock, 2001)

$$\%\text{Motor deficit} = [(\text{NEPT} - \text{EEPT})/\text{NEPT}]100 \quad (1)$$

The nociceptive withdrawal reflex (WRL) was adapted from the hotplate test developed by Masters et al. (1993) and described elsewhere (Luis et al., 2007, 2008a, b). Normal rats withdraw their paws from the hotplate within 4 s or less (Hu et al., 1997). The cutoff time for heat stimulation was set at 12 s to avoid skin damage to the foot (Varejao et al., 2003).

2.7.2. Sciatic functional index (SFI)

For SFI, animals were tested in a confined walkway measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end, as previously described (Luis et al., 2007, 2008a, b). Several measurements were taken from the footprints: (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). For SFI, all measurements were taken from the experimental (E) and normal (N) sides. The mean distances of three measurements were used to calculate the following factors:

Toe spread factor (TSF)=(ETS – NTS)/NTS

Intermediate toe spread factor (ITSF)=(EITS – NITS)/NITS

Print length factor (PLF)=(EPL – NPL)/ NPL

SFI was calculated as described by Bain et al., (1989) according to the following equation:

$$\begin{aligned}\text{SFI} = {} & -38.3(\text{EPL}-\text{NPL})/\text{NPL} + 109.5(\text{ETS}-\text{NTS})/\text{NTS} \\ & + 13.3(\text{EIT}-\text{NIT})/\text{NIT} - 8.8 = (-38.3 \times \text{PLF}) \\ & + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \end{aligned} \quad (2)$$

For SFI, an index score of 0 is considered normal and an index of −100 indicates total impairment. When no footprints were measurable, the index score of −100 was given (Dijkstra et al., 2000).

2.8. Sciatic nerve stereology

Nerve samples (10-mm-long sciatic nerve segments distal to the crush site and from un-operated controls) were processed for quantitative morphometry of myelinated nerve fibers (Raimondo et al., 2009). Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1 M Sorensen phosphate buffer for 6-8 hours and resin embedding was obtained following Glauerts' procedure (Scipio et al., 2008). Series of 2-μm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology was carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector were adopted using a protocol previously described (Geuna et al., 2000, 2004). Fiber density and total number of myelinated fibers were estimated together with fiber and axon diameter and myelin thickness.

2.9. Statistical analysis

Data of functional tests are reports as means and standard deviations (SD) at each time point, including pre-operatively, and each experimental group. Differences between time points and between groups were tested by two-way analysis of variance (ANOVA) using a mixed model of within (time of recovery) and between-subjects (experimental groups) factors. In cases of significant main effect of experimental group (between-subjects factor), pairwise comparisons were further conducted using the post hoc Tukey's HSD test using IBM SPSS statistics19 software package (SPSS, Inc.). For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milan, Italy). In RMN studies, comparison of data between different experimental groups was performed by One-way ANOVA followed by Newman–Keuls post-hoc test using GraphPad Prism Software, (San Diego California USA). In all cases differences at $p < 0.05$ were considered significant.

3. Results

3.1. Cytocompatibility of Vivosorb® membranes and confirmation of HMSCs differentiation into neuroglial-like cells and karyotype analysis.

Results obtained from epifluorescence technique are referred to measurements from undifferentiated HMSCs and neuroglial-differentiated HMSCs which correspond to $[Ca^{2+}]i$ from cells that did not begin the apoptosis process (data not shown). The undifferentiated HMSCs cultured on Vivosorb® membranes reached confluence and exhibited a normal star-like shape with a flat morphology in culture. In the presence of neurogenic medium, the formation of neuroglial-like cells was observed after 24 h. According to these results, it is reasonable to conclude that Vivosorb® membranes are a viable substrate for undifferentiated HMSCs culture or neuroglial-differentiated HMSCs adhesion, multiplication and differentiation.

The phenotype of HMSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell HMSCs isolated from Wharton's jelly of UC. HMSCs were tested for cell morphology, adherence rate and viability. Furthermore, for each cell lot of characterization was characterized by flow cytometry analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). The HMSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. During the expansion of cells long spindle-shaped and colonized the whole culturing surface was observed (Fig. 1A). After 96 h of culture in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches (Fig. 1B). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN in neuroglial-like differentiated MSCs. Undifferentiated HMSCs were negatively labeled to GFAP, GAP-43 and NeuN (Fig. 2A, C and E, respectively). After 96 h of differentiation the attained cells were positively stained for glial protein GFAP (Fig. 2B) and for the growth-associated protein GAP-43 (Fig. 2D). All nuclei of neuroglial-like cells were also labeled with the neuron specific nuclear protein NeuN (Fig. 2E) demonstrating successful differentiation of HMSCs in neuroglial-like cells.

Undifferentiated HMSCs exhibited a normal star-like shape with a flat morphology. After *in vitro* differentiation, HMSCs morphology changed into typical neuroglial-like pattern with multi-branches and secondary branches (Fig. 1B). Giemsa-stained cells of differentiated HMSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations

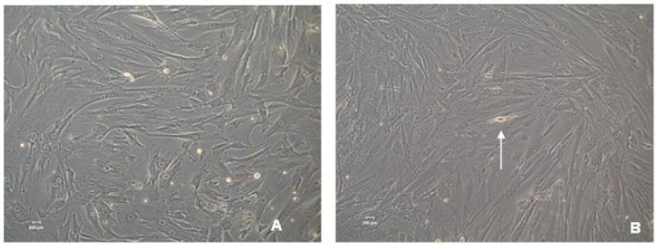

Fig. 1. HMSCs from Wharton's jelly exhibiting a mesenchymal-like shape with a flat polygonal morphology (A). After72 h of culture in neurogenic medium the cells became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches. (B) (Magnification: 100x).

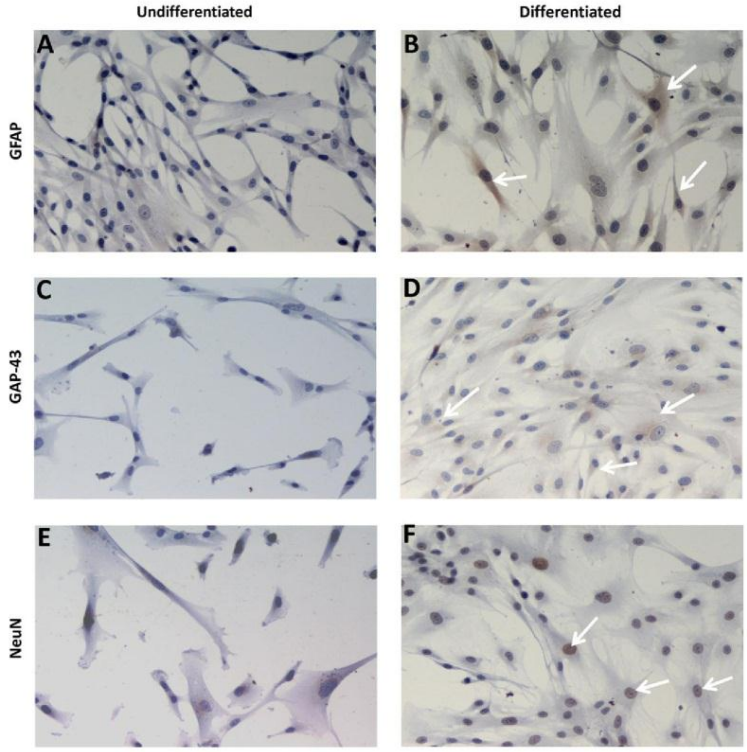

Fig. 2. Undifferentiated HMSC cells from the Wharton's jelly presenting a negative staining for: (A) GFAP which is a glial cell marker, (C) GAP-43 which is related with axonal outgrowth, and (E) NeuN which is a marker for nucleus of neurons. Neuroglial-like cells obtained from HMSCs *in vitro* differentiated with neurogenic medium exhibiting a positive staining for: (B) GFAP, (D) GAP-43, and (F) NeuN (Magnification: 200x).

were found demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures.

3.2. Glycolysis is stimulated during HMSCs expansion but not during differentiation

During the 96 h expansion of undifferentiated HMSCs, the glucose consumption was 4.4 ± 0.05 pmol/cell, while during differentiation the cells consumed 5.0 ± 1.1 pmol/cell. Interestingly, although the glucose consumption was very similar in both conditions after the 96 h cell culture, the glucose consumption rate was only similar between the 48 and 72 h. Undifferentiated HMSCs mostly consumed glucose in the first 48 h at a rate of 0.5 ± 0.004 pmol/h/cell/h, while during differentiation, the glucose consumption rate lowered to just 0.08 ± 0.01 pmol/h/cell/h. Between the 72 and 96 h of culture, the glucose consumption was also increased in undifferentiated cells (0.21 ± 0.04 pmol/h/cell) when compared with cells undergoing differentiation (0.08 ± 0.04 pmol/h/cell). As expected, the lactate production increased during HMSCs expansion. After 96 hours, the lactate production was 26 ± 1.1 pmol/cell during expansion, while during cell differentiation the total lactate production was 6.0 ± 0.6 pmol/cell. The lactate production rate during HMSCs expansion was increased during the first and last hours of expansion. In the first 48 h, the rate was 0.2 ± 0.03 pmol/h/cell and between the 72 and 96 h the rate was 0.5 ± 0.04 pmol/h/cell. Interestingly, during the 96 h of differentiation the lactate production rate was almost stable although between the 48 and 96 h of differentiation there was no production but a slight consumption of lactate.

3.3. Lactate and alanine metabolism is altered during differentiation of HMSCs

Alanine production was significantly decreased during HMSCs differentiation. During the 96 h expansion, the cells produced 6.9 ± 0.7 pmol/cell while after differentiation the alanine production was 2.2 ± 0.4 pmol/cell. During expansion and differentiation, the alanine was mostly produced during the first hours as seen by the alanine production rate. During the first 48 h, undifferentiated HMSCs produced alanine at a rate of 0.10 ± 0.003 pmol/h/cell while during differentiation, in that period, the alanine production rate was 0.02 ± 0.008 pmol/h/cell. The lactate/alanine ratio was significantly decreased after 48 and 72 h in undifferentiated HMSCs. The ratio decreases from 3.5 ± 0.3 at time zero of expansion to 2.1 ± 0.1 and 2.5 ± 0.3 in the following 48 and 72 h, respectively. After 96 h, HMSCs differentiated into neuroglial-like cells presented a significantly lower lactate/alanine ratio (2.6 ± 0.5) than undifferentiated cells (3.9 ± 0.2).

3.4. Functional analysis

3.4.1. Nociceptive function evaluated by withdrawal reflex latency (WRL)

The withdrawal reflex requires intact thermal and nociceptive innervations of the hindpaw. Early after sciatic axonotmesis, the

Table 1
Values in seconds (s) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), and every week after the surgical procedure until week-12. Results are presented as mean and standard deviation (SD). *N* corresponds to the number of rats within the experimental group ($N=6$).

	Week 0 Mean ± SD	**Week 1** Mean ± SD	**Week 2** Mean ± SD	**Week 3** Mean ± SD	**Week 4** Mean ± SD	**Week 5** Mean ± SD	**Week 6** Mean ± SD	**Week 7** Mean ± SD	**Week 8** Mean ± SD	**Week 10** Mean ± SD	**Week 12** Mean ± SD
Control	3.00 ± 0.63	3.00 ± 0.63	2.83 ± 0.41	2.67 ± 0.52	3.33 ± 0.52	3.3 ± 0.523	3.00 ± 0.00	3.17 ± 0.98	3.17 ± 0.41	2.50 ± 0.55	2.83 ± 0.41
Crush	1.67 ± 0.52	8.83 ± 4.22	6.83 ± 4.31	4.83 ± 3.25	4.50 ± 3.02	2.67 ± 0.82	2.33 ± 0.52	2.33 ± 0.52	2.33 ± 0.82	2.17 ± 0.41	2.00 ± 0.00
CrushPLC	3.22 ± 0.87	11.16 ± 0.90	9.46 ± 1.18	7.83 ± 1.41	6.83 ± 1.09	5.54 ± 0.51	4.80 ± 0.32	4.45 ± 0.26	4.20 ± 0.50	3.55 ± 0.29	3.04 ± 0.48
CrushCell	1.43 ± 0.53	10.86 ± 1.68	9.29 ± 2.29	7.43 ± 1.81	5.43 ± 2.15	3.43 ± 0.79	2.57 ± 0.53	2.14 ± 0.38	1.86 ± 0.38	1.57 ± 0.53	1.29 ± 0.49
CrushCellNonDiffPLC	3.70 ± 1,28	12.00 ± 0.00	10.83 ± 2.09	± 2.63	7.15 ± 2.99	6.41 ± 2.91	5.56 ± 1.57	4.90 ± 0.85	4.42 ± 1.30	3.48 ± 0.82	3.01 ± 0.59
CrushCellDiffPLC	3.68 ± 1.04	12.00 ± 0.00	11.80 ± 0.52	9.93 ± 2.06	8.60 ± 1.37	7.34 ± 0.50	5.66 ± 0.98	5.09 ± 0.75	4.69 ± 0.62	3.90 ± 0.63	3.01 ± 0.59

Group 1 = control (without any sciatic nerve injury), $n=6$.
Group 2 = crush (axonotmesis injury without any other intervention), $n=6$.
Group 3 = CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), $n=7$.
Group 4 = CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs), $n=7$.
Group 5 = CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of non-differentiated HMSCs), $n=6$.
Group 6 = CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), $n=7$.

Table 2
Values of motor deficit were obtained performing extensor postural thrust (EPT) test. This test has been performed preoperatively (week-0), and every week after the surgical procedure until week-12. Results are presented as mean and standard deviation (SD). *N* corresponds to the number of rats within the experimental group ($N=6$).

	Week 0 Mean ± SD	**Week 1** Mean ± SD	**Week 2** Mean ± SD	**Week 3** Mean ± SD	**Week 4** Mean ± SD	**Week 5** Mean ± SD	**Week 6** Mean ± SD	**Week 7** Mean ± SD	**Week 8** Mean ± SD	**Week 10** Mean ± SD	**Week 12** Mean ± SD
Control	0.01 ± 0.04	0.01 ± 0.06	0.00 ± 0.03	− 0.01 ± 0.04	− 0.01 ± 0.03	0.02 ± 0.06	0.03 ± 0.03	0.02 ± 0.03	0.00 ± 0.03	− 0.02 ± 0.07	0.00 ± 0.02
Crush	0.05 ± 0.06	0.84 ± 0.02	0.82 ± 0.05	0.71 ± 0.05	0.54 ± 0.06	0.42 ± 0.10	0.36 ± 0.07	0.15 ± 0.08	0.10 ± 0.13	0.02 ± 0.11	0.00 ± 0.06
CrushPLC	0.00 ± 0.17	0.84 ± 0.13	0.84 ± 0.14	0.75 ± 0.16	0.66 ± 0.15	0.52 ± 0.17	0.38 ± 0.11	0.32 ± 0.06	0.25 ± 0.05	0.16 ± 0.04	0.07 ± 0.09
CrushCell	0.01 ± 0.01	0.91 ± 0.01	0.84 ± 0.02	0.72 ± 0.02	0.54 ± 0.04	0.47 ± 0.03	0.35 ± 0.03	0.26 ± 0.02	0.15 ± 0.03	0.07 ± 0.01	0.03 ± 0.01
CrushCellNonDiffPLC	0.05 ± 0.12	1.00 ± 0.00	0.94 ± 0.02	0.86 ± 0.05	0.73 ± 0.19	0.45 ± 0.27	0.16 ± 0.12	0.18 ± 0.13	0.10 ± 0.08	0.04 ± 0.09	0.01 ± 0.12
CrushCellDiff PLC	0.05 ± 0.22	1.00 ± 0.00	0.94 ± 0.05	0.84 ± 0.07	0.65 ± 0.16	0.40 ± 0.23	0.20 ± 0.12	0.11 ± 0.09	0.05 ± 0.05	0.06 ± 0.07	0.02 ± 0.06

Group 1 = Control (without any sciatic nerve injury), $n=6$.
Group 2 = Crush (axonotmesis injury without any other intervention), $n=6$.
Group 3 = CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), $n=7$.
Group 4 = CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs), $n=7$.
Group 5 = CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of non-differentiated HMSCs), $n=6$.
Group 6 = CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), $n=7$.

withdrawal response could not be recorded and WRL values rose to the cutoff time of 12 s (Table 1). WRL progressively reduced approaching the normal values at week 12. There was a significant effect of time in WRL scores during the healing period [$F(10,330)=168.32$, $p=0.000$]. An overall main effect of kind of treatment was also noticed in WRL results during the healing time [$F(5,33)=22.786$, $p=0.000$]. WRL values were significantly different in groups treated with the PLC membrane, alone or in combination with undifferentiated and differentiated HMSCs (Group 5 – *CrushCellNonDifPLC*; Group 6 – *CrushCellDifPLC*; Group 3 – *CrushPLC*) with the groups not receiving the PLC membrane ($p<0.05$). In this case, however, the WRL values seem to indicate better functional recovery in the groups with the cellular system (Group 5 – *CrushCellNonDifPLC*; Group 6 – *CrushCellDifPLC*), demonstrating the positive results concerning the cellular system treatment (Table 1).

3.4.2. Motor performance by measuring extensor postural thrust (EPT)

The EPT measures the antigravity response when animals are lowered and the hindlimb is loaded. In control animals and before injury, EPT scores center around zero, showing similar forces in both sides. In the week following sciatic axonotmesis, EPT scores increased to near maximal values, indicating almost complete loss of contractile force in the affected hindlimb. EPT scores then recovered steadily in the following weeks to normal values. Therefore, a significant effect of time in EPT scores during the healing period was found [$F(10,330)=136.09$, $p=0.000$]. Also, differences in EPT scores between the experimental groups were significant [$F(5,33)=48.678$, $p=0.000$]. Further pairwise tests showed that the EPT values in the untreated axonotomized group (Group 2) were similar to the other axonotomized groups treated with different combinations of PLC membranes and cellular systems (Group 5 and Group 6). The groups treated with cells and PLC membrane (Group 5 and Group 6), however, presented significant differences in EPT values compared to the group treated with PLC membrane only (Group 3) ($p<0.05$), with positive results associated with the cellular system treatment. The group treated with infiltrated undifferentiated HMSCs (Group 4) presented worse recovery of EPT values, when compared to the group where these cells (Group 5 and Group 6) were applied locally, by means of a PLC membrane ($p<0.05$). There were no significant differences between the groups with PLC membranes plus undifferentiated and differentiated HMSCs (Group 5 and Group 6) (Table 2).

3.4.3. Sciatic functional index (SFI)

The SFI score demonstrated severe deficit in the 2 weeks following sciatic nerve crush in all experimental groups. In the following weeks, SFI values improved progressively ending up with values indistinguishable from control animals by week 12 of recovery. Therefore, a significant main effect of time was demonstrated by ANOVA [$F(10,330)=268.937$, $p=0.000$]. ANOVA also demonstrated a significant effect of group [$F(5,33)=52.632$, $p=0.000$]. Further pairwise comparisons located the group effect in the Group 4, with this group displaying delayed recovery of SFI compared to the Group 2 ($p=0.000$) and Group 2 ($p=0.004$) (Table 3).

3.5. Sciatic nerve stereology

Fig. 3 shows the histological appearance of the nerve fibers in the different experimental groups. As expected after a crush injury that does not interrupt the nerve continuity, axon regeneration occurred in all experimental groups. Results of the

Table 3
Sciatic Function Index (SFI) measured pre-operatively (week-0), and every week after the surgical procedure until week-12. An index score of 0 is considered normal and an index of 100 indicates total impairment. The measurements of the print length (PL), the toe spread (TS), and the intermediary toe spread (ITS), were taken from the experimental (*E*) and normal (*N*) sides. Results are presented as mean and standard deviation (SD). *N* corresponds to the number of rats within the experimental group ($N=6$).

	Week 0 Mean ± SD	Week 1 Mean ± SD	Week 2 Mean ± SD	Week 3 Mean ± SD	Week 4 Mean ± SD	Week 5 Mean ± SD	Week 6 Mean ± SD	Week 7 Mean ± SD	Week 8 Mean ± SD	Week 10 Mean ± SD	Week 12 Mean ± SD
Control	−12.43 ± 2.13	−9.17 ± 2.04	−8.59 ± 2.85	−8.54 ± 2.01	−8.06 ± 2.52	−9.92 ± 4.18	−7.59 ± 3.74	−7.16 ± 3.11	−7.84 ± 2.35	−10.03 ± 1.17	−8.80 ± 0.97
Crush	−13.15 ± 7.72	−69.80 ± 14.27	−71.00 ± 10.78	−39.85 ± 13.09	−28.88 ± 10.91	−28.95 ± 15.55	−26.13 ± 6.31	−13.67 ± 6.48	−10.62 ± 7.96	−6.72 ± 5.48	−5.33 ± 4.30
CrushPLC	−2.50 ± 18.55	−85.29 ± 9.53	−69.25 ± 10.17	−59.62 ± 14.80	−44.89 ± 6.62	−28.16 ± 6.44	−22.83 ± 6.79	−19.39 ± 6.98	−14.72 ± 7.36	−10.27 ± 4.26	−7.93 ± 2.78
CrushCell	−5.48 ± 3.40	−92.88 ± 9.55	−85.20 ± 10.56	−53.92 ± 11.22	−42.92 ± 6.99	−38.88 ± 6.65	−32.34 ± 8.41	−28.29 ± 7.64	−22.19 ± 4.69	−16.33 ± 2.47	−9.14 ± 3.67
CrushCellNonDifPLC	−1.69 ± 20.41	−81.08 ± 5.40	−83.79 ± 5.45	−62.74 ± 17.39	−26.63 ± 6.74	−23.25 ± 6.31	−13.58 ± 17.49	−11.30 ± 16.25	−19.62 ± 14.35	−10.02 ± 5.02	−5.47 ± 2.94
CrushCell Diff PLC	−12.48 ± 6.79	−76.94 ± 6.68	−74.16 ± 7.46	−56.42 ± 16.18	−34.44 ± 5.59	−27.08 ± 3.05	−26.61 ± 4.50	−24.09 ± 8.12	−18.19 ± 8.35	−10.85 ± 3.30	−8.56 ± 4.87

Group 1 = Control (without any sciatic nerve injury), $n=6$.
Group 2 = Crush (axonotmesis injury without any other intervention), $n=6$.
Group 3 = CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), $n=7$.
Group 4 = CrushCell (axonotmesis lesion infiltrated with a suspension of 1250–1500 HMSCs), $n=7$.
Group 5 = CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of non-differentiated HMSCs), $n=6$.
Group 6 = CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), $n=7$.

stereological analysis of regenerated nerve fibers are reported in Table 4. Statistical analysis showed that all axonotmesis groups have a significantly ($p < 0.05$) higher number and density of regenerated nerve fibers compared to control. By contrast, in all axonotmesis groups, nerve fibers showed a significantly ($p < 0.05$) smaller diameter, of both axon and fiber, and myelin thickness. As far as numerical differences among axonotmesis groups are concerned, statistical analysis did not reveal any significant ($p > 0.05$) difference for any of the morphological predictors of nerve recovery except for myelin thickness that was significantly ($p < 0.05$) higher in Group 5 and Group 6.

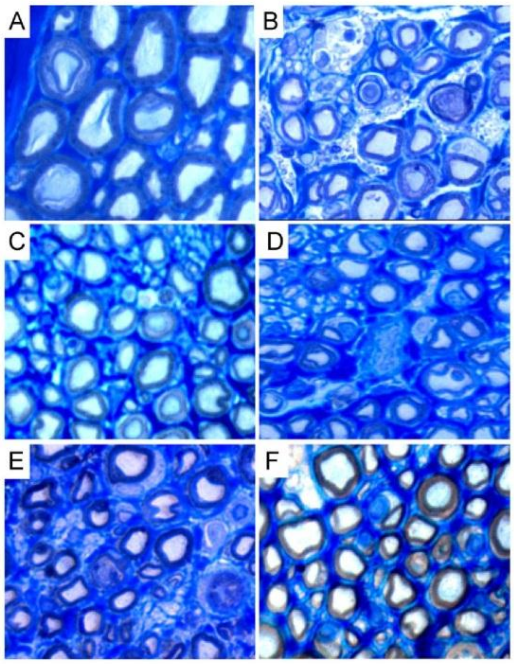

Fig. 3. Histological appearance of the regenerated nerve fibers in the different experimental groups: Control (A), Crush (B), CrushPLC (C), CrushCell (D), CrushCellNonDifPLC (E), CrushCellDifPLC (F) (Magnification: 1000x).

4. Discussion

Peripheral nerve tissue engineering associates biomaterials to cellular systems, able to differentiate into neuroglial-like cells, which might improve motor and sensory recovery. SCs, MSCs, embryonic stem cells, marrow stromal cells are the most studied support cells candidates. The cellular systems implanted into the injured nerve may produce growth factors or extracellular matrix (ECM) molecules, or may modulate the inflammatory process, to improve nerve regeneration (Amado et al., 2010; Gu et al., 2011; Luis et al., 2007, 2008a; Simoes et al., 2010). To implant cultured cells into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). It can also be performed by pre-adding the cells to the neural scaffold via injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve (Maurício et al., 2011).

The majority of natural biomaterials used in clinical applications (for instance, peripheral nerve injuries) such as hyaluronic acid, collagen, and gelatin are derived from animal sources. In spite of thorough purification methods, these materials bear the inherent risk of transfer of viral diseases and may cause immunological body reactions while synthetic biomaterials are not associated with these risks (Maurício et al., 2011). Results obtained from epifluorescence technique that measure the $[Ca^{2+}]i$ and the cell culture morphology of undifferentiated HMSCs and neuroglial-differentiated HMSCs cultured on Vivosorb® membranes evidence that this biomaterial is a viable substrate for undifferentiated HMSCs culture or neuroglial-differentiated HMSCs adhesion, multiplication and differentiation.

Our data showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients (Maurício et al., 2011). PLC becomes hydrophilic by water uptake, which increases the permeability of the polymer. This is important in the control of nutrient and other metabolite transport to the surrounding healing tissue. A few weeks after implantation, the mechanical strength gradually decreases and loss of molecular weight occurs as a result of the hydrolysis process. In approximately 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PCL has the advantage of not creating an acidic and

Table 4
Histomorphometrical assessment of normal (Group 1—Control) and regenerated sciatic nerves submitted to a standardized sciatic nerve crush injury with non-serrated clamp (week-12 post-traumatic). Results are presented as mean and standard deviation (*SD*). *N* corresponds to the number of rats within the experimental group ($N=6$).

Group	Fiber density (*N*/mm2)	Fiber *N*umber (*N*)	Fiber diameter (μm)	Axon diameter (μm)	Myelin thickness (μm)
Control	15,905 ± 287	7666 ± 190	6.66 ± 0.12	4.26 ± 0.07	1.19 ± 0.03
Crush	20,109 ± 1,232	10,644 ± 423	4.99 ± 0.19	3.48 ± 0.10	0.76 ± 0.05
CrushPLC	23,349 ± 4,278	11,532 ± 1,086	4.55 ± 0.36	3.36 ± 0.17	0.60 ± 0.10
CrushCell	20,200 ± 4,971	9806 ± 2,695	5.31 ± 0.69	3.74 ± 0.49	0.78 ± 0.10
CrushCellNondiffPLC	20,945 ± 1,162	10,909 ± 1,584	5.11 ± 0.42	3.39 ± 0.25	0.86 ± 0.11
CrushCellDiffPLC	23,593 ± 5,611	9640 ± 1,784	4.93 ± 0.43	3.25 ± 0.37	0.84 ± 0.05

Group 1=Control (without any sciatic nerve injury), $n=6$.
Group 2=Crush (axonotmesis injury without any other intervention), $n=6$.
Group 3=CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), $n=7$.
Group 4=CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs), $n=7$.
Group 5=CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of non-differentiated HMSCs), $n=6$.
Group 6=CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), $n=7$.

potentially disturbing micro-environment, which is favorable to the surrounding tissue (Luis et al., 2007).

Extra-embryonic tissues, as stem cell reservoirs, offer many advantages over both embryonic and adult stem cell sources. Extra-embryonic tissues, collectively known as the afterbirth, are routinely discarded at parturition, so little ethical controversy attends the harvest of the resident stem cells populations. Most significantly, the comparatively large volume of extra-embryonic tissues and easy manipulation hypothetically increases the number of stem cells that can be isolated (Marcus and Woodbury, 2008). The UC contains two arteries and one vein protected by a proteoglycan rich connective tissue called Wharton's jelly. Within the abundant extracellular matrix of Wharton's jelly resides a recently described stem cell population. In average, 400,000 cells can be isolated per UC, which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow. *In vitro*, Wharton's jelly MSCs cells are capable of differentiation to multiple mesoderm cell types including skeletal muscle and neurons (Fu et al., 2006; Wang et al., 2004). Generation of clinically important dopaminergic neurons has also been reported (Fu et al., 2006). HMSCs isolated from Wharton's jelly can be easily and ethically obtained and processed compared with embryonic or bone marrow stem cells. These stem cells may be a valuable source in the repair of the peripheral nervous system with capacity to differentiate into neuroglial-like cells (Fu et al., 2006; Yang et al., 2008). The transplanted HMSCs were also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Fu et al., 2006; Wang et al., 2004). Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration (Luis et al., 2008a; Maurício et al., 2011). The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration (Amado et al., 2008, 2010; Luis et al., 2008a; Maurício et al., 2011). Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis (Amado et al., 2008, 2010; Luis et al., 2008a; Maurício et al., 2011) and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplastic origin. In this study, we used a PLC membrane to deliver undifferentiated and *in vitro* differentiated HMSCs from the UC Wharton jelly and we compared this delivery approach with direct infiltration of these undifferentiated HMSCs in suspension, in the rat sciatic nerve axonotmesis model. The cellular systems implanted into the injured nerve may produce growth factors or ECM molecules, or may modulate the inflammatory process, to improve nerve regeneration or even replaced the injured neural and SCs (Amado et al., 2008, 2010; Luis et al., 2008a; Maurício et al., 2011). The differentiated HMSCs karyotype could not be established, once no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated HMSCs previously determined and published elsewhere, excluded the presence of neoplastic signs, thus supporting the suitability of our cell culture and differentiation procedures. This concern also resulted from our previous experience with N1E-115 neoplastic cell line and the negative results we obtained in the treatment of axonotmesis and neurotmesis injuries (Amado et al., 2008, 2010; Luis et al., 2008b; Maurício et al., 2011). Nevertheless, our cultured undifferentiated HMSCs showed normal morphology when inspected with an inverted microscope. These cells presented a star-like shape with a flat morphology, characteristic of the MSCs.

A consequence of cell metabolism during *in vitro* expansion and differentiation is that culture conditions are constantly changing. The comprehension and optimization of the expansion and differentiation process of HMSCs will contribute to maximization of cell yield, reduction of cell culture and a decrease of total process costs (Gong et al., 2009; Kirouac and Zandstra, 2008). In culture conditions, cells consume mostly glucose as substrate for the generation of cellular energy (ATP). A high rate of glucose consumption is necessary for cell growth and maintenance. In fact, it has been proposed that cells undergoing high proliferation rates rely essentially on glycolysis to generate ATP, producing considerable amounts of lactate (Vander Heiden et al., 2009). This process, known as Warburg effect, has been described to occur in HMSCs during expansion (Dos Santos et al., 2010; Funes et al., 2007). Our results obtained from the *in vitro* testing of this cellular system show that during 96 h expansion, the undifferentiated HMSCs consumed glucose and produce, as expected, high concentration of lactate as a metabolic sub-product which is consistent with the Warburg effect and glycolysis stimulation. It was also described that MSCs do not require oxidative phosphorylation to survive (Dos Santos et al., 2010; Funes et al., 2007) instead, hypoxia prolongs the lifespan of these cells, increases their proliferative capacity and reduces differentiation(Fehrer et al., 2007). Several studies in recent years investigated the biological activity of HMSCs and their *in vitro* differentiation in neuroglial-like cells (Fu et al., 2004; Mitchell et al., 2003). In this study, HMSCs were *in vitro* differentiated with neurogenic medium. After 96 h the regular mesenchymal-like shape of the cells changed and the cells became exceedingly long. Morphologically it was observed the formation of neuroglial-like cells after differentiation which were positively stained for the typical specific neuronal markers (Choong et al., 2007; Fu et al., 2004; Mitchell et al., 2003) such as the GFAP, the GAP-43 and the NeuN attesting a clear successful differentiation. The morphologic and biochemical characteristics of neuroglial-like cells are already described but the mechanism by which stem cells differentiate into neuroglial-like cells is still unknown. In our experimental conditions, HMSCs that undergo differentiation in neuroglial-like cells, consumed significantly less glucose and produced significantly less lactate than HMSCs that undergo expansion. These major differences allow us to conclude that during HMSCs differentiation in neuroglial-like cells the glycolytic process, which proved to be the crucial metabolic mechanism during HMSCs expansion, is switched to oxidative metabolism. Several factors, such as inhibition of oxidative phosphorylation due to defects in mitochondrial DNA, dysfunctional Krebs cycle, or inactivation of p53 have been suggested to contribute to the glycolytic switch occurr during cellular expansion and differentiation (Funes et al., 2007). Recently, it has been shown that umbilical-cord derived mesenchymal stem cells adapt their oxygen consumption and energy metabolism to the available oxygen concentrations (Lavrentieva et al., 2010). In our conditions, HMSCs consumed glucose at higher rates during the first 48 h of expansion. After that period the glucose consumption rate decreases although there was no reduction in lactate production rate. Interestingly, during HMSCs differentiation in neuroglial-like cells, lactate production rate is significantly lower and between 72 and 96 h, there was no lactate production rate but rather consumption. The conversion of pyruvate in lactate is an NADH-dependent reduction but pyruvate may also be converted into alanine via transaminase reaction, through the enzyme alanine

aminotransaminase (Yang et al., 2002). Surprisingly, during HMSCs expansion, the alanine production significantly increased but the same was not noted during differentiation in neuroglial-like cells. However, the lactate/alanine ratio was lower at 48 and 72 h during HMSCs expansion than at the same periods of differentiation. After 96 h this ratio was decreased in differentiated cells. The appearance of more alanine than lactate in the media of neuroglial-like cells can be associated with a reduced redox cytosolic state (low ratio NADH/NAD+) which may point to a role of oxidative stress during the differentiation of HMSCs in neuroglial-like cells, however further investigation is needed to prove this suggestion. Our results show clear evidences that HMSCs expansion is dependent of glycolysis while their differentiation in neuroglial-like cells requires the switch of the metabolic profile to oxidative metabolism. Also important may be the role of oxidative stress during this process.

Here we also tested the efficacy of our combined biomaterial and cellular system approach in the *in vivo* treatment of sciatic nerve crush injury. Following transection, axons show staggered regeneration and may take substantial time to actually cross the injury site and enter the distal nerve stump (Brushart et al., 2002). Although delayed axonal elongation might be caused by growth inhibition originating from the distal nerve itself, growth-stimulating influences may overcome axons stagger. More robust and fast nerve regeneration is expected to result in better reinnervation and functional recovery. As a potential source of growth promoting signals, HMSCs transplantation is expected to have a positive outcome. Our results showed that the use of either undifferentiated or differentiated HMSCs enhanced the recovery of sensory and motor function. The observation that in both cell-enriched experimental groups myelin sheath was thicker, suggest that HMSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Geuna et al., 2009).

5. Conclusion

We conclude that HMSCs isolated from the Wharton's jelly of the UC delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis.

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Ethical approval

This work is original in that it has not been published before or submitted for publication elsewhere, and will not be submitted elsewhere before a decision has been taken as to its acceptability in this Journal where you are Editor. Each author meets the criteria for authorship and assumes the corresponding responsibility. In this study, laboratory animals were used. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed.

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1.4. Conclusions

The association of biomaterials with cellular systems in peripheral nerve tissue engineering might improve motor and sensory recovery. SCs, MSCs, embryonic stem cells, marrow stromal cells are the most studied support cells candidates. It is believed that cellular systems implanted into the injured nerve may produce growth factors or extracellular matrix (ECM) molecules, or may even modulate the inflammatory process, to improve nerve regeneration (Amado *et al.*, 2010; Gu *et al.*, 2011).

Most of the natural biomaterials used in peripheral nerve injuries are derived from animal sources. These materials carry the risk of transfer of viral diseases and may cause immunological body reactions while synthetic biomaterials do not have these risks (Maurício *et al.*, 2011). Cell culture morphology of undifferentiated MSCs and neuroglial-like differentiated MSCs cultured on PLC membranes, evidence that this biomaterial is a viable substrate for undifferentiated HMSCs culture or neuroglial-like differentiated HMSCs adhesion, multiplication and differentiation.

In conclusion, for the *in vitro* studies performed with MSCs from PromoCell:

- The karyotype analysis of the MSCs cell line derived from Wharton's jelly of human umbilical cord demonstrated that this cell line in terms of number and structure of the somatic and sexual chromosomes has no neoplastic characteristics and is stable during the cell culture procedures. Also, the morphologic characteristics of these cells in culture, observed in an inverted microscope, were perfectly normal. These cells presented a star-like shape with a flat morphology, characteristic of MSCs.
- The differentiated MSCs karyotype could not be established, once no dividing cells were obtained at passage 5, which is in agreement with the degree of differentiation.
- The cell culture morphology of undifferentiated MSCs and differentiated neuroglial-like MSCs cultured on PLC membranes evidence that this biomaterial is a feasible substrate for undifferentiated MSCs culture adhesion, multiplication and differentiation.
- After 96 h of culture in neurogenic medium the regular mesenchymal-like shape of the MSCs changed and the cells became exceedingly long. Morphologically it was possible to observe the formation of neuroglial-like cells after differentiation

which were positively stained for typical specific neuronal markers such as the GFAP, the GAP-43 and the NeuN confirming a clear successful differentiation.

In conclusion, for the axonotmesis *in vivo* studies:

- The significant improvement of axonal regeneration obtained in crushed sciatic nerves surrounded by chitosan type III membranes suggests that this material may not just work as a simple mechanical scaffold but instead may work as an inducer of nerve regeneration.
- The use of human MSCs associated or not to the hybrid chitosan membranes showed positive effects concerning the functional recovery (evaluated by EPT and WRL tests), most likely due to the modulation of the inflammatory process during the Wallerian degeneration and the production of growth factors.
- Employment of human MSCs alone or in combination with chitosan type III membrane after nerve crush injury provides a slight advantage in comparison to untreated controls. On the other hand, our results confirmed that chitosan type III membranes alone may represent a very promising clinical tool in peripheral nerve reconstructive surgery.
- The use of both undifferentiated and differentiated MSCs improved the recovery of sensory and motor function. In both cell enriched experimental groups myelin sheath was thicker, suggesting that MSCs might exert a positive effects on SCs, the elementary key in Wallerian degeneration and following axonal regeneration.

2. Neurotmesis injuries

2.1. Introduction

Neurotmesis is the most frequent type of injury in peripheral nervous damages, which is characterized by a complete disorganization of the nerve anatomy. Axonal regrowth is impossible, but spontaneous recovery is extremely difficult without surgical intervention (Robinson, 2000), even after recovery it's still not satisfactory.

Even with all the scientific advances and clinical knowledge, the full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remain the principle goal of regenerative biology. (Mackinnon *et al.*, 2001; *Battiston et al.*, 2005). In the peripheral nervous system, nerves can spontaneously regenerate without any treatment if the nerve continuity is maintained, which happens in axonotmesis, whereas more severe type of injuries must be surgically treated by direct end-to-end surgical reconnection of the damaged nerve ends (Mackinnon *et al.*, 1985; Amado *et al.*, 2008; Ronchi *et al.*, 2009).

Whenever tension-free suturing is possible direct repair is the choice. Nevertheless, when there is a nerve gap resulting from loss of nerve tissue, an autologous nerve graft is a common choice. However there are some disadvantages with the worst being donor site morbidity (Mackinnon *et al.*, 2001). Entubulation with autologous non-nervous tissues or other kind of biomaterials could be a good alternative, where donor site morbidity is no longer a problem and tissue availability could be endless. Many studies have shown the importance of peripheral nerve repair with the use of entubulation technique whenever neurotmesis occurs. In these situations nerves will grow and regenerate from proximal towards distal nerve stump, preventing this way ingrowth of fibrous tissue and neuroma formation (Gärtner *et al.*, 2013).

In the following studies 20 weeks was the time for follow-up recovery. The recovery time is crucial once it is proportional to the severity of the lesion. Based on previous works (Luís *et al.*, 2007b; Luís *et al.*, 2008a) 20 weeks seemed to be the ideal time for these studies.

Concerning the peripheral nerve regeneration, it is important to identify an appropriate vehicle to the local application of the MSCs in the injury site. This vehicle must be biocompatible and may be a hydrogel or a membrane that supports the adhesion, expansion and survival of the cellular system (Maurício *et al.*, 2011; Gärtner *et al.*, 2013). The resorption of a biomaterial should be adjusted to the regeneration process, which depends on its molecular weight, composition, crystal structure and thermal history. When associating a biomaterial to a cellular system it is also important to

determine properties such as hydrophobicity, surface charge and surface rugosity to establish its ability to support adhesion and cell growth (Harley *et al.*, 2006).

2.2
In preparation

Use of poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells for promoting nerve regeneration in a neurotmesis rat model: In vitro and in vivo analysis

Use of poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly of the umbilical cord for promoting nerve regeneration in a neurotmesis rat model: *in vitro* and *in vivo* analysis.

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) have the potential to differentiate into a variety of tissues and might be responsible for turnover and maintenance of adult tissue. It is also known that these cells migrate to damaged site, especially to hypoxia, inflammation and apoptosis spots, modifying the surrounding microenvironment, promoting repair with functional improvements. It has been suggested that MSCs have a reparative influence through the paracrine effect on the survivor Schwann cells and by modulating the regenerative process and the Wallerian degeneration.

Methods/Design: In the present study the effects of enwrapping the site of end-to-end rat sciatic nerve repair with a poly(DL-lactide-ε-caprolactone) (Vivosorb®) membrane associated or not with undifferentiated human umbilical cord matrix MSCs (HMSCs) or HMSCs pre-differentiated neuroglial-like cells, was investigated. Motor and sensory functional recovery was evaluated throughout a healing period of 20 weeks using extensor postural thrust (EPT), withdrawal reflex latency (WRL) and ankle kinematic analysis. Histological observation was carried out on regenerated nerve fibers.

Discussion: *In vitro* investigation showed the formation of typical neuroglial-like cells after differentiation, which was positively stained for the typical specific neuroglial markers. Results showed that enwrapment of the rapair site with a Vivosorb® membrane, with undifferentiated HMSCs or neuroglial-like cell enrichment, did not lead to any significant improvement in most of functional and stereological predictors of nerve regeneration that were assessed, with the exception of EPT which showed a significantly better recovery after employment of undifferentiated HMSCs enriched membrane. HMSCs isolated from the Wharton's jelly of the UC delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves induced by neurotmesis injuries with loss of nervous tissue which enables an end-to-end suture or a grafting procedure. When neurotmesis injury can be surgically reconstructed with an epineural end-to-end suture without tension or by grafting, the addition of a PLC membrane associated with a cellular system is not mandatory for a substantial improvement in functional and morphological recoveries. Therfore it can be concluded that this particular type of nerve tissue engineering approach has very limited effects on nerve regeneration after sciatic end-to-end nerve reconstruction or grafting in the rat.

KEY WORDS: neurotmesis, end-to-end suture, stem cells, mesenchymal stem cells, neuroglial-like cells, nerve regeneration, Wharton jelly.

1. INTRODUCTION

Traumatic injuries in Central and Peripheral Nervous (PN) System are the ones with less success in terms of functional motor recovery and the ones that give more incapacity across the world. In the absence of surgical intervention, functional regeneration is restricted due to scars, neuroma formation, mismatched fibers or extensive splitting of the re-growing axons; moreover peripheral regeneration is often associated to neuropathic pain, which is very limiting for the patient even more than the incomplete functional recovery [1]. The functional outcome gets worse with the degree of injury, regeneration is weaker if a nerve gap or neuroma has been developed leading to functional impairment [1, 2]. Temporal delay from time of trauma to the surgical repair is also an important factor for poor functional outcome due to irreversible regional muscle atrophy [3]. Neurotmesis is the most common type of injury in peripheral nervous injuries, and it is characterized by a complete disorganization of nerve anatomy, so, axonal regrowth is impossible and spontaneous recovery is extremely difficult without surgical intervention [4]. Despite advances in scientific and clinical knowledge, it is still an important aim to have a full understanding of axonal recovery and organ reinnervation in a nerve defect [5, 6] and new therapeutic approaches associated to physiotherapy besides surgical reconstruction are needed in order to achieve a satisfactory functional recovery [7]. Whenever tension-free suturing is possible direct end-to-end repair is the choice. Nevertheless, when there is a nerve gap resulting from loss of nerve tissue, an autologous nerve graft is a common choice. However there are some disadvantages with the worst being donor site morbidity [6]. Many studies have shown the importance of peripheral nerve repair using a tube-guide whenever neurotmesis occurs. In these situations nerves will grow and regenerate from proximal towards distal nerve stump, preventing the ingrowth of fibrous tissue and neuroma formation [8]. The development of cell-based therapies began a new age in tissue regeneration [8]. Regeneration is a physical process through which remaining tissues organize themselves to replace and repair injured or missing tissues *in vivo*. Stem cells from different sources are much probably the golden key for regenerative medicine [9]. Among these, mesenchymal stem cells (MSCs) have become one of the most interesting targets due to their well-known characteristics. MSCs have a high plasticity, proliferative and differentiation capacity together with attractive immunosuppressive properties [8]. Once there have been many approaches for MSCs culture, the Mesenchymal and Tissue Stem Cell Committee, of International Society for cellular therapy (ISCT), recommended several standards to define MSCs [10]. Much

evidence demonstrates that the therapeutic effects of these cells do not simply lie on the capacity to repair damage tissue, but on the ability to modulate surrounding tissue by secretion of various factors and activation of endogenous progenitor cells [11, 12]. In many studies MSCs have proven to modify the surrounding environment promoting functional repair decreasing inflammation and immune response [13]. Increasing evidence demonstrate that MSCs may play an important role in tissue regeneration through the secretion of soluble trophic factors which enhance and modulate the repair process by paracrine activation of surrounding cells. The use of cellular systems is a rational approach for delivering neurotrophic factors at the nerve lesion site [8]. MSCs can be isolated from several tissues, including bone marrow, skin, periosteum, amniotic fluid, umbilical cord blood and matrix and adipose tissue [8]. Bone marrow represents the most frequently used tissue source. MSCs from bone marrow have been applied for cell based therapies; however, represent several disadvantages like limited number of MSCs available, the heterologous and non-consistent nature of bone marrow preparations, the possibility of donor site morbidity, as well as decreased number of MSCs along the adult life. For these reasons, it is imperative to identify alternative and more primordial MSCs sources that would allow a safe and controlled *ex vivo* expansion for potential allogeneic application. Umbilical cord tissue-derived MSCs exhibit a neuronal phenotype [14-17] and have potential utility in treatment of neurodegenerative diseases [18, 19], indicating the versatility of this cell source. Interestingly, these cells are negative for the class II major histocompatibility complex (MHC), and have low expression MHC class I expression [16], making them a potential cell source for MSC-based therapies. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses [7].

MSCs have been precious for cell-based therapies, the evidence is that there are 142 clinical trials going on and 25 are based on umbilical cord MSCs (clinical trials.gov).

To avoid excessive connective tissue formation when re-joining nerve ends a bridging material is used. The best option is to use an autologous nerve; nevertheless this carries several limitations; like co-morbidity at donor site, loss of sensation, neuroma and scar formation, as well as insufficient revascularization of the graft [1, 20]. Whenever autografts are not possible, nerve conduits may be an option, as a good alternative for gaps between 3-10mm [21].

The ideal biomaterial for nerve conduit must fulfil several biological and physicochemical requirements and may be biological or synthetic. Such requirements

are; biocompatibility, biodegradability, permeability to ions and metabolites for the revascularization of the regenerated nerve, biomechanical and surface properties should enable and modulate the cellular system adhesion [7]. Among synthetic biodegradable materials, poly(DL-lactide-ε-caprolactone) (PLC) attracted particular attention to our research group [22-26]. The biodegradation rate of PLC is estimated to be approximately 16 months and the degradation products of PLC are less acidic, comparable, for instance with poly(L-lactide): poly(glycolide) (PLGA), which may cause less damage to the surrounding tissue, and they are transparent facilitating the correct positioning of the nerve stumps. Previous *in vitro* studies have shown that PLC membranes and tube-guides are biocompatible with nerve cells and may facilitate nerve cell attachment, differentiation and growth [22, 23, 27, 28]. Also, *in vivo* studies have demonstrated that PLC might improve morphological and functional recoveries, in axonotmesis and neurotmesis injuries of the rat sciatic nerve. The structure of the polymer was still well preserved, after 20 weeks, in nerves repaired by PLC [22, 23]. Shin and colleagues reported in 2009 a study in which they compared 3 synthetic bioabsorbable conduits with a reversal autograft in sciatic nerve repair. One of the synthetic absorbable conduits was also a PLC tube-guide which showed the best results comparing with the ones obtained with autograft [29]. In the present study we proposed to test *in vivo* the therapeutic value of human umbilical cord matrix MSCs (HMSCs) undifferentiated or differentiated into neuroglial-like cells together with a poly(DL-lactide-ε-caprolactone) (Vivosorb®) membrane as nerve guide, to promote nerve regeneration on rat sciatic nerve neurotmesis injury surgically reconstructed with an epineural end-to-end suture or by autologous grafting. Functional recovery after peripheral nerve injury and repair is rarely satisfactory [30-34]. In case of loss of substance a nerve autograft procedure, using commonly expendable sensory nerves, is required [35, 36]. However, nerve autograft leads to donor site morbidity and secondary sensory deficit [37]. Alternatives to peripheral nerve grafts include cadaver nerve segment allografts, end-to-side neurorraphy and entubulation by means of autologous non nervous tissues such as vein and muscles [38-44].

2. MATERIALS AND METHODS

2.1. Poly(DL-lactide-ε-caprolactone) (PLC) membranes

Poly(DL-lactide-ε-caprolactone) (PLC) membranes (Vivosorb®) were purchased from Polyganics BV, Groningen, Netherlands (FS01-006/20 Lot: FSA2009092311). Vivosorb® is a flexible bioresorbable polymer film, made of Poly(DL-lactide-ε-

caprolactone) copolymer which presents retention of mechanical strength for up to 10 weeks throughout the critical healing period.

2.2. Cell Culture and *in vitro* differentiation of HMSC from Wharton's jelly umbilical cord

Human MSC from Wharton's jelly umbilical cord (HMSCs) were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreserved cells were cultured and maintained in a humidified atmosphere with 5% CO_2 at 37ºC. Mesenchymal stem cell medium (PromoCell, C-28010) was replaced every 48 hours. At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. HMSCs at a concentration of 2500 cell/ml were cultured and after 24 hours cells exhibited 30-40% confluence. Differentiation was induced with MSC neurogenic medium (PromoCell, C-28015). Medium was replaced every 24 hours during 3 days. The formation of neuroglial-like cells was observed after 24 hours in an inverted microscope (Zeiss, Germany).

2.3. Immunocytochemistry

At passage 3, HMSCs were trypsinized, washed and re-suspended in mesenchymal stem cell medium (PromoCell, C-28010) at a concentration of $1x10^5$cell/ml. HMSCs were fixed with paraformaldehyde at 4ºC for 15 min and washed with distilled water before permeabilization in 0.5% Triton-X100. Non-specific binding was blocked using blocking solution (PBS containing 1% bovine serum albumin (BSA)) for 1 hour at room temperature. HMSCs were then incubated 2 hours at room temperature with primary antibodies of rabbit anti-growth associated protein-43 (GAP-43, 1:200) (Chemicon, AB5220), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500) (Chemicon, AB5804) and mouse anti-neuronal nuclei (NeuN, 1:100) (Chemicon, MAB377). After washing, HMSCs were incubated 15 minutes with secondary antibodies goat anti-rat IgG (Millipore, AP136P) and goat anti-rabbit IgG (Millipore, 12-348MN). After several washes in PBS, HMSCs were incubated with horseradish peroxidase (HRP)-coupled streptavidin for 10 min. DAB (diaminobenzidine) served as chromogen.

2.4. Surgical procedure

All the animal testing procedures are in conformity with the Directive 2010/63/EU of the European Parliament and with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986

(86/609/EEC). Humane end points were followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000). For the *in vivo* testing, *Sasco Sprague* adult rats (Charles River Laboratories, Barcelona, Spain) were divided in groups of 6 animals each. All animals were housed in a temperature and humidity controlled room with 12-12 hours light / dark cycles, two animals per cage (Makrolon type 4, Tecniplast, VA, Italy), and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking in account human endpoints for animal suffering and distress. Animals were housed for two weeks before entering the experiment. The neurotmesis injury was carried out with the animals placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. The surgery procedure was the one previously described by Luís et al [22]. The right sciatic nerve was exposed and a transection (neurotmesis) injury was performed, immediately above terminal nerve ramification, with a straight microsurgical scissor. A group of 6 animals was used as control without any sciatic nerve injury (Group 1 – *Control*). In Group 2 the injured nerve was left without any repair intervention (Group 2 – *Gap*). In Group 3, immediate cooptation with 7/0 monofilament nylon epineural sutures of the 2 transected nerve endings was performed (Group 3 – *End-to-End*). In Group 4, the two endings of nerve transection were immediately sutured with a 7/0 monofilament nylon suture, and enwrapped in a PLC (Vivosorb®) membrane covered with a monolayer of non-differentiated HMSCs (Group 4 – *End-to-EndPLCcellnonDif*), in Group 5, the two endings of nerve transection were immediately sutured as the previous group and enwrapped in a PLC (Vivosorb®) membrane covered with a monolayer of differentiated HMSCs (Group 5 – *End-to-EndPLCcellDif*). For the last three groups the sciatic nerve was bisected immediately above the terminal nerve ramification and at a10mm distal point. The resulting nerve graft, with a length of 10mm, was inverted 180º and sutured with 7/0 monofilament nylon. One group was used as control for the graft (Group 6 – *Graft*), in another group the graft was enwrapped in a PLC (Vivosorb®) membrane covered with a monolayer of non-differentiated HMSCs (Group 7 –*GraftPLCcellnonDif*) and in the last one graft was enwrapped in a PLC (Vivosorb®) membrane covered with a monolayer of differentiated HMSCs (Group 8 – *GraftPLCcellDif*) (Figure 1). No local or systemic signs of rejection or foreign body were observed in the experimental animals transplanted with PLC

membranes and HMSCs (undifferentiated and differentiated). There was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period of 20 weeks after surgical procedure.

2.5. Functional assessment

All animals were tested pre-operatively (week 0), and every week until week 12 and every 2 weeks until the end of follow-up time (20 weeks). Animals were gently handled, and tested in a quiet environment to minimize stress levels.

2.5.1. Motor performance and nociceptive function

The EPT was originally proposed by Thalhammer and collaborators, in 1995 [45] as a part of the neurological recovery evaluation in the rat after sciatic nerve injury. For this test, the entire body of the rat, excepting the hind-limbs, was wrapped in a surgical towel. Supporting the animal by the thorax and lowering the affected hind-limb towards the platform of a digital balance, elicits the EPT. As the animal is lowered to the platform, it extends the hind-limb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance (model TM560; Gibertini, Milan, Italy) was recorded. The same procedure was applied to the contra-lateral, unaffected limb. Each EPT test was repeated 3 times and the average result was considered. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into an equation (Equation 1) to derive the functional deficit (varying between 0 and 1), as described by Koka and Hadlock, in 2001 [46].

$$\text{Motor Deficit} = (\text{NEPT} - \text{EEPT}) / \text{NEPT} \qquad \text{(Equation 1)}$$

To assess the nociceptive withdrawal reflex (WRL), the hotplate test was modified as described by Masters and collaborators [47]. The rat was wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate at 56ºC (model 35-D, IITC Life Science Instruments, Woodland Hill, CA). WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. Normal rats withdraw their paws from the hotplate within 4.3 s or less [48]. The affected limbs were tested 3 times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies were averaged to obtain a final result [49]. If there was no paw withdrawal

after 12 s, the heat stimulus was removed to prevent tissue damage, and the animal was assigned the maximal WRL of 12 s [50, 51].

2.5.2. Kinematic Analysis

Ankle kinematics was carried out before nerve injury (week 0), and at the 20-week follow-up time. Animals walked on a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus was adjusted to the size of the rats during the experiments. The rats' gait was video recorded at a rate of 300 Hz images per second (Casio Exilim Pro EX-F1, Japan). The camera was positioned at the track half-length where gait velocity was steady, and 1 m distant from the track obtaining a visualization field of 14 cm wide. The video images were stored in a computer hard disk for latter analysis using an appropriate software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, USA). 2-D biomechanical analysis (sagittal plan) was carried out applying a two-segment model of the ankle joint, adopted from the model firstly developed by [25]. The rats' ankle angle was determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (θ^{o}) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points were identified: initial contact (IC), Opposite Toe off (OT), and Heel Rise (HR) and toe-off (TO) [25, 52, 53] and were time normalized for 100% of step cycle. The normalized temporal parameters were averaged over all recorded trials. A total of six walking trials for each animal with stance phases lasting between 150 and 400 ms were considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) [25].

2.6. Histology and Scanning electron microscopy (SEM)

Nerve samples (10-mm-long sciatic nerve segments distal to the crush site and from un-operated controls) were processed for histological analysis of myelinated nerve fibers [54]. Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding was obtained following Glauerts' procedure [55]. Series of 2-µm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Histological observation was carried

out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

Prior to scanning electron microscopy (SEM) analysis, the HMSCs cultured on PLC discs and the PLC tube-guide without HMSCs (Figures 2C and 2D) were first fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) for 2h at 4^{o}C. Afterwards, the PLC samples with and without the HMSCs were dehydrated using graded ethanol solutions from 60 % to 100%, 5 minutes each, and subjected to critical point drying. Finally, the samples were mounted on aluminum stubs using double-side adhesive tape and sputter coated with gold/palladium thin film, using the SPI Module Sputter Coater equipment for 100 seconds and with a 15mA current. The SEM / EDS exam was performed using a high resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M.

2.7. Statistical analysis

The general linear model procedure was employed to undertake two-way mixed factorial ANOVA. The design included two between-subjects variables with either two conditions (grafting vs. non-grafting) or three conditions (no-cells; undifferentiated cells; differentiated cells) and one within-subjects factor consisting on the repeated measures of the outcome variables across the recovery time. Mauchly's test was used to test sphericity and correction of the degrees of freedom was performed with the Greenhouse-Geiser's epsilon whenever this assumption could not be demonstrated. Simple planned contrasts (General Linear Model, simple contrasts) were used to compare pooled data across all experimental groups during recovery with pre-operative data. Pairwise comparisons between groups were carried out by employing the HSD Tukey's test. All statistical procedures were performed by using the statistical package SPSS (version 14.0, SPSS, Inc). All data in this study is presented as mean ± standard deviation of the mean (SD).

3. RESULTS

3.1. Confirmation of HMSCs differentiation into neuroglial-like cells

The phenotype of HMSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell mesenchymal stem cells isolated from Wharton's jelly of umbilical cord. HMSCs were tested for cell morphology, adherence rate and viability. Furthermore, each cell lot was characterized by flow cytometry analysis for a

comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). The HMSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion the cells became long spindle-shaped and colonized the whole culturing surface (Figure 2A). After 96 hours of culture in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches (Figure 2B). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN in neuroglial-like differentiated MSCs. Undifferentiated HMSCs were negatively labeled to GFAP, GAP-43 and NeuN. After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP (Figure 3B) and for the growth-associated protein GAP-43. All nuclei of neuroglial-like cells were also labeled with the neuron specific nuclear protein NeuN demonstrating successful differentiation of HMSCs in neuroglial-like cells [56].

Undifferentiated HMSCs exhibited a normal star-like shape with a flat morphology (Figures 2A and 2D). After *in vitro* differentiation, HMSCs morphology changed into typical neuroglial-like pattern with multi-branches and secondary branches (Figure 2B). Giemsa-stained cells of differentiated HMSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations were found demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures [56].

3.3. Functional analysis

3.3.1. Nociceptive function evaluated by withdrawal reflex latency (WRL)

Table 1 presents the data for the WRL during recovery. As expected, in the weeks immediately following sciatic nerve transection and repair, animals were unable to respond to the hot stimulus, which is compatible with complete loss of thermal and nociceptive sensitivity of the sole of the foot. Regaining of the foot withdrawal response started at the second week following sciatic nerve transection and repair. The WRL significantly improved during the 20-weeks recovery time although never reaching the normal values even at the end of the 20-weeks recovery (simple contrasts, 20 weeks vs. pre-injury; P=0.035). Differences in WRL recovery were found both as a result of grafting [$F_{(1,31)}$ = 7.765; P = 0.009] and as a result of HMSCs application [$F_{(2,31)}$ =

14.112; P = 0.000]. Therefore, faster and better recovery in WRL response occurred in animals treated with sciatic nerve end-to-end repair (*End-to-End*, *End-to-EndPLCcellnonDif*, and *End-to-EndPLCcellDif* groups) compared to those receiving a graft (*Graft*, *GraftPLCcellnonDif*, and *GraftPLCcellDif* groups). Also, faster WRL responses were exhibited by animals that did not receive either undifferentiated (P = 0.001) or differentiated (P = 0.000) HMSCs (*End-to-End* group and *Graft* group). No recovery in WRL response existed in untreated animals following sciatic nerve transection (*Gap* group).

3.3.2. Motor performance by measuring extensor postural thrust (EPT)

Animals presented a severe motor deficit immediately following sciatic nerve transection demonstrated by almost complete loss of EPT response in the affected hind limb (Table 2). EPT response in the affected side improved partially in all treated animals with time and a significant motor deficit still existed at week 20 after sciatic nerve injury (simple contrasts, 20 weeks vs. pre-injury; P = 0.000). Significantly different EPT responses existed during recovery from sciatic nerve transection and repair due to the use of HMSC cells [F(2,31) = 28.778; P = 0.000] but not due to grafting [F(1,31) = 0.271; P = 0.606]. Pairwise comparisons confirmed the presence of significant differences in EPT response following sciatic nerve injury between animals that were not treated with HMSC cells and those treated with undifferentiated (P = 0.016) and differentiated (P = 0.000) cells. Also, significant differences were found in EPT response between animals treated with undifferentiated and differentiated HMSC cells (P = 0.000). Interestingly, the EPT response following sciatic nerve transection and repair displayed a significant interaction between the type of nerve repair (i.e. direct end-to-end repair or grafting) and the application of stem cells [F(2,31) = 4.910; P = 0.014]. Analysis of groups' mean values regarding the EPT response throughout the entire 20 weeks period suggests that undifferentiated cells, but not differentiated cells, enhanced EPT recovery in grafting-treated animals, thus partially counteracting the negative effect of nerve grafting in functional recovery, comparing with direct end-to-end sciatic nerve repair.

3.4. Kinematic Analysis

The angle and angular velocity of the ankle joint (Figures 3A and 3B) during the stance phase of step cycle were measured in the affected side at the end of the 20-weeks recovery period following sciatic nerve neurotmesis. The same measures were also

collected in uninjured control rats. Ankle angle was similar in all groups of animals recovering from sciatic nerve neurotmesis and for all instant of stance phase considered (Figure 3A). Similarly, no differences could be found for ankle angle at any studied time instant of the stance phase between groups of animals recovering from sciatic nerve neurotmesis and control animals, irrespectively of nerve treatment employed (Figure 3A).

Regarding ankle angular velocity, univariate ANOVA found a significant effect for the kind of treatment for sciatic nerve neurotmesis at HR [$F(6,33) = 10.414$; $P = 0.000$] and TO [$F(6,33) = 2.542$; $P = 0.039$], but not at IC [$F(6,33) = 1.311$; $P = 0.135$] and OT [$F(6,33) = 1.776$; $P = 0.280$]. At HR, pairwise differences for values of ankle velocity existed between the *Gap* group and *End-to-EndPLCcellDiff*, *GraftPLCcellnonDiff,* and *GraftPLCcellnonDiff* groups ($P > 0.05$). At this time point, ankle velocity in all groups of sciatic nerve-injured animals was significantly different from the uninjured group. The same results were obtained at TO, with significant differences in the values of ankle velocity between uninjured animals and all the remaining groups. In this case, however, no differences were noticed for ankle velocity between any of sciatic nerve neurotmesis groups (Figure 3B).

3.5. Sciatic nerve histology

Histological analysis on semi thin sections showed that nerve fiber regeneration occurred in all repaired nerves. In comparison to controls (Figure 4), in all repaired nerves regenerated fibers showed small axons with thin myelin sheaths and microfasciculation (Figure 5). Microfasciculation was more evident in the graft repair groups (Figures 5C and 5D) in comparisons to *End-to-End* group (Figures 5A and 5B). From a histological point of view, the comparison between treatment with undifferentiated and differentiated HMSC did not show clear differences both after end-to-end (Figures 5A and 5B) and graft (Figures 5C and 5D) nerve reconstruction.

4. DISCUSSION

Tissue engineering focusing on the *in vitro* fabrication of autologous, living tissues with the potential of regeneration is a promising scientific and clinical field. Peripheral nerve regeneration should include a multidisciplinary team able to develop biomaterials, to develop cell therapies, and to elaborate *in vitro* analysis and pre-clinical trials concerning animal welfare and the most appropriate animal model before the clinical trials and clinical application approval [57]. Transected peripheral nerves can

regenerate whenever a connection is available between the proximal and distal severed stumps and, when no substance loss occurs, surgical treatment consists in direct end-to-end suturing of the nerve ends [30-34]. However, in spite of the progress of microsurgical nerve repair, the outcome of nerve reconstruction is still far from being optimal [58]. Since during regeneration axons require neurotrophic support, they could benefit from the presence of a cellular system capable of responding to stimuli of the local environment during axonal regeneration. In case of loss of substance a nerve autograft procedure, using commonly expendable sensory nerves, is required [36, 59, 60]. However, nerve autograft leads to donor site morbidity and secondary sensory deficit [37]. Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorraphy and entubulation by means of autologous non nervous tissues such as vein and muscles [38, 40, 44, 61, 62]. Therefore one of the scientist's challenges over the last thirty years has been to find an alternative to the autologous nerve graft [61]. Employment of a nerve conduit (i.e. a tubular structure designed to bridge the gap of a sectioned nerve, protect the nerve from the surrounding tissue, and guide the regenerating axons into the distal nerve stump) is the most popular alternative to nerve autografts; yet, conduits can also play an important role as a vehicle for neurotrophic factors and cellular systems [23, 24, 27, 38, 40, 41, 43, 44, 62].

In a previously published study the therapeutic value of HMSCs on rat sciatic nerve after axonotmesis injury associated to the same PLC membrane (Vivosorb®), was evaluated. Also, *in vitro* characterization of the cellular system was previously assessed concerning nuclear magnetic resonance (NMR) analysis, immunocytochemistry, and intracellular ionic calcium concentration measurements using the epifluorescence technique where the cell viability was indirectly evaluated, when cultured on PLC discs (Vivosorb®). During HMSCs expansion and differentiation in neuroglial-like cells, the culture medium analysis by nuclear magnetic resonance was performed in order to evaluate the metabolic profile. It was also important to confirm the possibility of PLC membranes to support the expansion of HMSCs, to correlate the HMSCs ability to differentiate and survival capacity in the presence of the Vivosorb® membrane, the $[Ca^{2+}]i$ of undifferentiated HMSCs or neuroglial-differentiated HMSCs was determined by the epifluorescence technique using the Fura-2AM probe. The Vivosorb® membrane proved to be adequate to be used as a scaffold associated to undifferentiated HMSCs or neuroglial-differentiated HMSCs, so the experimental work could continue furthermore, with more serious injuries, namely, neurotmesis

without loss of nervous tissue. *In vitro* investigation demonstrated the formation of typical neuroglial cells after differentiation which was positively stained for the typical specific neuroglial markers such as the GFAP, the GAP-43 and NeuN. NMR showed clear evidence that HMSCs expansion is glycolysis-dependent but their differentiation requires the switch of the metabolic profile to oxidative metabolism. *In vivo* studies showed enhanced recovery of motor and sensory function in animals treated with transplanted undifferentiated and differentiated HMSCs that was accompanied by an increase in myelin sheath [56]. Crush injuries are appropriate to investigate the cellular and molecular mechanisms of peripheral nerve regeneration, and to assess the role of different factors in the regeneration process [63]. Nerve crush injury is also a well-established model in experimental regeneration studies to investigate the impact of various pharmacological treatments [27, 64, 65] and should be used before testing therapeutic approaches in a more serious lesion, like neurotmesis. The present study intended to confirm the ability of PLC membranes together with undifferentiated and differentiated HMSCs to promote nerve regeneration, improving the functional and morphological recoveries even when grafting or and epineural end-to-end suture is possible. Results regarding the EPT response throughout the entire 20 weeks period suggest that undifferentiated cells, but not differentiated cells, enhanced EPT recovery in grafting-treated animals, thus partially counteracting the negative effect of nerve grafting in functional recovery, comparing with direct end-to-end sciatic nerve repair. Ankle angle was similar in all groups of animals recovering from sciatic nerve neurotmesis and for all instant of stance phase considered. Similarly, no differences could be found for ankle angle at any instant time of the stance phase between groups of animals recovering from sciatic nerve neurotmesis and control animals, irrespectively of nerve treatment employed. Regarding ankle angular velocity a significant effect for the kind of treatment for sciatic nerve neurotmesis at HR and OT, was found. At HR, differences for values of ankle velocity existed between the *Gap* group and *End-to-EndPLCcellDiff*, *GraftPLCcellnonDif,* and *GraftPLCcellnonDif* groups. At this time point, ankle velocity in all groups of sciatic nerve-injured animals was significantly different from the uninjured group. The same results were obtained at TO, with significant differences in the values of ankle velocity between uninjured animals and all the remaining groups. In this case, however, no differences were noticed for ankle velocity between any of sciatic nerve neurotmesis groups. Histological analysis showed that nerve fiber regeneration occurred in all repaired nerves. Also all repaired nerves regenerated fibers showed small axons with thin myelin sheaths and

microfasciculation was more evident in the graft repair groups in comparisons to *End-to-End* groups. From a histological point of view, the comparison between treatment with undifferentiated and differentiated HMSC did not show clear differences both after end-to-end and graft nerve reconstruction. Results showed that enwrapment of the repaired site with a Vivosorb® membrane, with undifferentiated HMSCs or neuroglial-like cell enrichment, did not lead to any significant improvement in most of functional and stereological predictors of nerve regeneration that we assessed, with the exception of EPT which recovered significantly better after employment of undifferentiated HMSCs enriched membrane. HMSCs isolated from the Wharton's jelly of the UC delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves after neurotmesis injuries with loss of nervous tissue that enables an end-to-end suture or a grafting procedure. When the neurotmesis injury can be surgically reconstructed with an epineural end-to-end suture without tension or by grafting, the addition of a PLC membrane associated with a cellular system is not mandatory for a substantial improvement in functional and morphological recoveries. It can thus be concluded that this particular type of nerve tissue engineering approach has very limited effects on nerve regeneration after sciatic end-to-end nerve reconstruction or grafting in the rat.

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CONFLICT OF INTEREST: There is no conflict of interest.

ETHICAL APPROVAL: This work is original in that it has not been published before or submitted for publication elsewhere, and will not be submitted elsewhere before a decision has been taken as to its acceptability in this Journal where you are Editor. Each author meets the criteria for authorship and assumes the corresponding responsibility.

In this study, laboratory animals were used. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European

Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed.

5. FIGURE AND TABLE LEGENDS

Figure 1 - Rat sciatic nerve injury and surgical reconstruction

Surgical approach to the rat's sciatic nerve (A); Nerve reconstruction after induced neurotmesis lesion: Surgical reconstruction where the neurotmesis injury reconstructed with an end-to-suture is enwrapped in a PLC (Vivosorb®) membrane (B); Nerve gap of 10mm (C); and End-to-end epineural suture (D).

Figure 2 – Cell and membrane images

Monocultures of HMSCs from Wharton's jelly over PLC membrane exhibiting a mesenchymal-like shape with a flat polygonal morphology (A). Monocultures of HMSCs HMSCs from Wharton's jelly over PLC membrane after 72h of culture in neurogenic medium, differentiated HMSCs became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches (B) (Magnification: 100x). SEM image of PLC tube-guide (Magnification: 250x) (C). SEM image of HMSCs cultured over a PLC disc (Magnification: 1000x).

Figure 3 – Kinematic plots

Kinematic plots in the sagittal plane for angular position (º) (A) and for angular velocity (º/s) (B) as it moves through the stance phase, obtained at week-20 after the neurotmesis injury. The mean of each group is plotted.

Figure 4 – Histological image

Histological appearance of a normal rat sciatic nerve (Magnification: 1000x).

Figure 5 – Histological images

Histological appearance of regenerated nerve fiber treated with undifferentiated and differentiated HMSC: *End-to-endPLCcellnonDif* (A), *End-to-endPLCcellDif* (B), *GraftPLCcellnonDif* (C), *GraftPLCcellDif* (D) (Magnification: 1000X).

Table 1 – Withdrawal Reflex Latency results

Values in seconds (s) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively

(week-0), at week 1 and 2 and after every two weeks until the end of the 20-week follow-up time. Results are presented as mean and standard deviation (SD). N corresponds to the number of rats within the experimental group.

Table 2 – Extensor Postural Thrust results
Values of Motor Deficit were obtained performing Extensor Postural Thrust (EPT) test. This test has been performed pre-operatively (week-0), at week 1 and 2 and after every two weeks until the end of the 20-week follow-up time. Results are presented as mean and standard deviation (SD). N corresponds to the number of rats within the experimental group.

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TABLES

Table 1 – Withdrawal Reflex Latency results

	Week 0	Week 1	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16	Week 18	Week 20
Control												
1A	3,00	12,00	4,00	4,00	10,00	5,00	8,00	12,00	5,00	7,00	5,00	4,00
1B	2,00	12,00	5,00	12,00	5,00	2,00	10,00	2,00	4,00	2,00	2,00	3,00
1C	2,00	12,00	12,00	8,00	5,00	3,00	4,00	4,00	4,00	6,00	4,00	4,00
1D	4,00	12,00	2,00	3,00	4,00	5,00	2,00	6,00	4,00	4,00	4,00	2,00
1E	2,00	12,00	6,00	2,00	10,00	2,00	5,00	2,00	2,00	6,00	2,00	2,00
1F	2,00	12,00	12,00	12,00	12,00	3,00	3,00	3,00	4,00	4,00	2,00	2,00
1G	1,00	12,00	12,00	8,00	10,00	4,00	2,00	2,00	2,00	3,00	2,00	2,00
Mean ± SD	2,29±0,95	12,0±0,00	7,57±4,31	7,00±4,12	8,00±3,21	3,43±1,27	4,86±3,08	4,43±3,64	3,57±1,13	4,57±1,81	3,00±1,29	2,71±0,95
Gap												
2A	2,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00
2B	1,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00
2C	2,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00
2D	2,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00	12,00
Mean ± SD	1,75±0,50	12,00±0,00	12,00±0,00	12,00±0,00	12,00±0,00	48,00±0,00	12,00±0,00	12,00±0,00	12,00±0,00	12,00±0,00	12,00±0,00	12,00±0,00
End-to-end												
3A	3,00	13,00	4,00	4,00	10,00	5,00	8,00	13,00	5,00	7,00	5,00	4,00
3B	3,00	13,00	5,00	13,00	5,00	3,00	10,00	3,00	4,00	3,00	3,00	3,00
3C	3,00	13,00	13,00	8,00	5,00	3,00	4,00	4,00	4,00	6,00	4,00	4,00
3D	4,00	13,00	3,00	3,00	4,00	5,00	3,00	6,00	4,00	4,00	4,00	3,00
3E	3,00	13,00	6,00	3,00	10,00	3,00	5,00	3,00	3,00	6,00	3,00	3,00
3F	3,00	13,00	13,00	13,00	13,00	3,00	3,00	3,00	4,00	4,00	3,00	3,00
3G	1,00	13,00	13,00	8,00	10,00	4,00	3,00	3,00	3,00	3,00	3,00	3,00
Mean ± SD	3,39±0,95	13,0±0,00	7,57±4,31	7,00±4,13	8,00±3,31	3,43±1,37	4,86±3,08	4,43±3,64	3,57±1,13	4,57±1,81	3,00±1,39	3,71±0,95
End-to-endPLCcellnonDif												
4A	3,89	13,00	13,00	13,00	11,05	13,00	13,00	9,34	7,39	7,58	5,64	3,93
4B	4,08	10,86	10,77	8,44	7,35	7,10	5,64	5,44	5,03	4,34	4,10	3,84
4C	3,35	13,00	13,00	7,88	9,13	8,59	8,03	7,39	7,40	6,55	6,49	5,93
4D	4,36	13,00	13,00	13,00	13,00	13,00	13,00	5,36	8,03	7,61	7,65	7,80
4E	3,74	13,00	13,00	10,00	4,60	13,00	6,40	3,58	3,71	5,34	5,88	6,31
4F	5,40	13,00	13,00	13,00	13,00	13,00	10,15	10,49	7,36	5,73	5,30	4,60
Mean ± SD	3,79±1,03	11,81±0,47	11,80±0,50	10,39±1,90	9,35±3,95	10,63±3,30	9,04±3,77	6,90±3,64	6,45±1,69	6,18±1,33	5,84±1,19	5,40±1,56
End-to-endPLCcellDif												
5A	4,03	13,00	13,00	13,00	13,00	9,75	13,00	9,49	9,81	7,99	9,35	3,87
5B	3,31	4,39	4,75	6,36	3,39	4,83	3,94	3,63	4,73	6,38	7,75	4,34
5C	3,19	13,00	13,00	13,00	13,00	6,68	13,00	9,33	9,73	5,79	8,31	11,54
5D	3,06	13,00	13,00	13,00	13,00	11,06	10,84	10,11	9,60	9,30	8,19	6,75
5E	3,54	13,00	13,00	13,00	11,37	9,51	5,34	7,45	4,43	5,33	5,13	5,90
5F	3,43	13,00	13,00	13,00	13,00	13,00	13,00	13,00	13,00	13,00	11,56	11,54
Mean ± SD	3,91±0,73	10,73±3,15	10,79±3,96	11,04±3,34	10,43±3,51	8,97±3,71	9,34±3,73	8,67±3,87	8,38±3,08	7,76±3,53	8,38±3,10	7,33±3,43
Graft												
6A	3,00	13,00	13,00	13,00	13,00	13,00	5,00	4,00	3,00	4,00	4,00	4,00
6B	3,00	13,00	13,00	13,00	13,00	13,00	8,00	5,00	3,00	3,00	3,00	3,00
6C	3,00	13,00	13,00	13,00	13,00	13,00	4,00	4,00	3,00	3,00	3,00	3,00
6D	3,00	13,00	13,00	13,00	13,00	13,00	13,00	10,00	7,00	7,00	13,00	13,00
6E	1,00	13,00	13,00	13,00	13,00	13,00	6,00	6,00	4,00	3,00	3,00	3,00
6F	3,00	13,00	13,00	13,00	13,00	13,00	5,00	4,00	4,00	3,00	3,00	3,00
Mean ± SD	1,83±0,41	13,00±0,00	13,00±0,00	13,00±0,00	13,00±0,00	13,00±0,00	6,67±3,94	5,50±3,35	4,00±1,55	3,83±1,60	4,50±3,73	4,67±3,61
GraftPLCcellnonDif												
7A	3,88	13,00	13,00	11,37	11,06	11,04	10,39	9,03	8,77	8,19	7,64	6,30
7B	4,03	13,00	13,00	13,00	13,00	13,00	11,74	11,14	6,44	5,60	5,88	5,39
7C	3,36	13,00	13,00	13,00	13,00	9,54	8,34	6,67	6,34	6,13	5,73	5,53
7D	3,87	13,00	13,00	13,00	13,00	11,96	8,81	7,37	7,47	6,56	5,77	5,38
7E	3,18	13,00	13,00	13,00	13,00	11,75	9,66	8,83	8,30	6,83	5,93	5,34
7F	4,48	13,00	13,00	13,00	13,00	9,35	6,93	6,16	5,58	5,03	4,73	4,80
Mean ± SD	3,47±0,84	13,00±0,00	13,00±0,00	11,88±0,30	11,84±0,38	10,94±1,31	9,39±1,69	8,30±1,84	7,13±1,33	6,39±1,10	5,59±0,94	5,41±0,46
GraftPLCcellDif												
8A	3,13	13,00	13,00	13,00	13,00	13,00	11,74	10,17	9,05	8,33	7,15	5,31
8B	3,50	13,00	13,00	13,00	11,86	9,64	9,45	8,73	7,85	7,31	5,98	5,58
8C	3,67	13,00	13,00	13,00	13,00	13,00	13,00	13,00	13,00	13,00	11,56	10,35
8D	3,64	13,00	13,00	13,00	13,00	10,86	10,43	9,74	8,33	6,33	6,03	5,63
8E	3,53	13,00	13,00	13,00	13,00	13,00	11,73	10,81	9,18	8,51	8,04	7,61
8F	3,63	13,00	13,00	13,00	13,00	9,57	7,37	6,47	5,89	6,03	5,80	4,87
Mean ± SD	3,18±0,51	13,00±0,00	13,00±0,00	13,00±0,00	11,98±0,06	11,01±1,18	10,45±1,80	9,65±1,90	8,73±3,00	8,07±3,16	7,43±3,30	6,54±3,10

Table 2 – Extensor Postural Thrust results

	Week 0	Week 1	Week 3	Week 4	Week 6	Week 8	Week 10	Week 13	Week 14	Week 16	Week 18	Week 30
Control												
1A	0,07	0,90	0,80	0,73	0,68	0,56	0,54	0,54	0,64	0,48	0,43	0,50
1B	0,07	0,93	0,83	0,70	0,73	0,66	0,57	0,50	0,45	0,40	0,40	0,40
1C	0,08	0,90	0,85	0,88	0,83	0,76	0,57	0,50	0,53	0,44	0,38	0,40
1D	0,07	0,90	0,91	0,84	0,83	0,73	0,57	0,39	0,55	0,38	0,34	0,40
1E	0,09	0,97	0,97	0,69	0,60	0,56	0,51	0,46	0,45	0,38	0,38	0,39
1F	0,06	0,77	0,86	0,83	0,78	0,73	0,63	0,57	0,55	0,38	0,38	0,40
1G	0,07	0,87	0,89	0,81	0,78	0,76	0,70	0,47	0,45	0,33	0,43	0,50
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08	0,74±0,08	0,68±0,09	0,58±0,06	0,49±0,06	0,53±0,07	0,40±0,05	0,39±0,03	0,43±0,05
Gap												
2A	0,08	0,90	0,93	0,93	0,93	0,98	0,87	0,84	0,84	0,91	0,96	0,94
2B	0,09	0,89	0,90	0,90	0,95	0,89	0,87	0,90	0,89	0,95	0,90	0,95
2C	0,07	0,95	0,90	0,87	0,75	0,85	0,90	0,83	0,80	0,79	0,84	0,86
2D	0,08	0,98	0,90	0,93	0,95	0,90	0,91	0,95	0,89	0,87	0,95	0,90
Mean ± SD	0,08±0,01	0,93±0,04	0,91±0,01	0,90±0,03	0,89±0,10	0,91±0,05	0,89±0,03	0,88±0,06	0,86±0,04	0,88±0,07	0,91±0,06	0,91±0,04
End-to-end												
3A	0,07	0,90	0,80	0,73	0,68	0,56	0,54	0,54	0,64	0,48	0,43	0,50
3B	0,07	0,93	0,83	0,70	0,73	0,66	0,57	0,50	0,45	0,40	0,40	0,40
3C	0,08	0,90	0,85	0,88	0,83	0,76	0,57	0,50	0,53	0,44	0,38	0,40
3D	0,07	0,90	0,91	0,84	0,83	0,73	0,57	0,39	0,55	0,38	0,34	0,40
3E	0,09	0,97	0,97	0,69	0,60	0,56	0,51	0,46	0,45	0,38	0,38	0,39
3F	0,06	0,77	0,86	0,83	0,78	0,73	0,63	0,57	0,55	0,38	0,38	0,40
3G	0,07	0,87	0,89	0,81	0,78	0,76	0,70	0,47	0,45	0,33	0,43	0,50
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08	0,74±0,08	0,68±0,09	0,58±0,06	0,49±0,06	0,53±0,07	0,40±0,05	0,39±0,03	0,43±0,05
End-to-endPLCcellnonDif												
4A	0,03	0,97	0,94	0,98	0,97	0,88	0,80	0,84	0,79	0,73	0,50	0,13
4B	0,06	0,97	0,98	0,98	0,99	0,99	0,85	0,85	0,80	0,61	0,45	0,50
4C	0,36	0,99	0,93	0,98	0,91	0,91	0,90	0,83	0,87	0,64	0,60	0,38
4D	0,14	0,97	0,86	0,98	0,93	0,94	0,90	0,88	0,83	0,64	0,60	0,08
4E	0,03	0,95	0,88	0,88	0,90	0,83	0,77	0,76	0,75	0,57	0,39	0,15
4F	-0,05	0,90	0,87	0,98	0,95	0,99	0,79	0,80	0,64	0,51	0,31	0,03
Mean ± SD	0,08±0,11	0,96±0,03	0,91±0,05	0,96±0,04	0,94±0,03	0,93±0,06	0,84±0,06	0,83±0,04	0,78±0,08	0,63±0,07	0,46±0,14	0,19±0,17
End-to-endPLCcellDif												
5A	0,13	0,95	0,97	0,97	0,97	1,00	0,98	0,99	0,98	0,99	0,99	0,87
5B	0,03	0,93	0,98	0,94	0,93	0,98	0,75	0,89	0,55	0,59	0,75	0,33
5C	-0,08	0,96	0,97	0,96	0,94	0,98	0,99	0,89	0,76	0,73	0,61	0,66
5D	0,10	0,98	0,98	0,99	0,98	0,83	0,86	0,70	0,80	0,68	0,50	0,48
5E	0,00	0,93	0,94	0,93	0,95	0,84	0,77	0,53	0,40	0,40	0,79	0,60
5F	0,46	0,98	0,98	0,99	0,98	0,98	0,66	0,99	0,98	0,98	0,98	0,98
Mean ± SD	0,11±0,19	0,11±0,19	0,97±0,03	0,96±0,03	0,96±0,03	0,94±0,08	0,84±0,13	0,83±0,19	0,75±0,33	0,75±0,33	0,77±0,33	0,64±0,37
Graft												
6A	0,07	0,83	0,87	0,78	0,78	0,81	0,73	0,73	0,69	0,60	0,50	0,50
6B	0,08	0,89	0,91	0,93	0,75	0,75	0,60	0,64	0,53	0,46	0,54	0,53
6C	-0,08	0,93	0,94	0,88	0,78	0,65	0,63	0,63	0,50	0,45	0,57	0,54
6D	0,00	0,91	0,85	0,83	0,77	0,73	0,58	0,68	0,64	0,54	0,57	0,54
6E	0,09	0,93	0,89	0,83	0,77	0,74	0,63	0,64	0,69	0,64	0,50	0,54
6F	0,00	0,89	0,87	0,89	0,78	0,65	0,66	0,67	0,53	0,50	0,54	0,46
Mean ± SD	0,03±0,07	0,90±0,04	0,89±0,03	0,85±0,05	0,77±0,01	0,73±0,06	0,64±0,05	0,66±0,03	0,60±0,09	0,53±0,08	0,54±0,03	0,53±0,03
GraftPLCcellnonDif												
7A	0,03	0,97	0,98	0,99	0,99	0,89	0,76	0,56	0,59	0,53	0,16	0,31
7B	0,34	0,99	0,99	0,99	0,98	0,85	0,70	0,70	0,67	0,48	0,05	0,13
7C	-0,03	0,99	0,98	0,99	0,99	0,94	0,73	0,67	0,48	0,40	0,33	0,33
7D	0,33	0,99	0,98	0,99	0,99	1,00	0,88	0,50	0,60	0,38	0,35	0,13
7E	0,01	0,97	0,98	0,99	0,99	0,84	0,59	0,38	0,39	0,37	0,33	0,08
7F	-0,43	0,98	0,99	0,99	0,98	0,98	0,85	0,70	0,66	0,44	0,03	0,01
Mean ± SD	0,01±0,34	0,98±0,01	0,98±0,01	0,99±0,00	0,99±0,01	0,93±0,07	0,75±0,11	0,59±0,13	0,57±0,11	0,40±0,11	0,19±0,14	0,13±0,08
GraftPLCcellDif												
8A	-0,15	0,90	0,98	0,99	0,98	0,98	0,94	0,84	0,73	0,64	0,60	0,53
8B	0,18	0,98	0,98	0,99	0,99	0,98	0,98	0,93	0,77	0,49	0,38	0,35
8C	0,15	0,98	0,97	0,98	0,98	0,98	0,98	0,94	0,93	0,87	0,76	0,77
8D	-0,06	0,99	0,98	0,99	0,98	0,98	0,99	0,98	0,85	0,69	0,63	0,66
8E	0,39	0,98	0,98	0,98	0,98	0,98	0,98	0,88	0,63	0,58	0,61	0,50
8F	-0,35	0,98	0,99	0,99	0,99	0,99	0,93	0,76	0,68	0,35	0,36	0,34
Mean ± SD	0,34±0,04	0,97±0,03	0,98±0,01	0,99±0,01	0,98±0,01	0,98±0,00	0,97±0,03	0,89±0,08	0,76±0,11	0,59±0,31	0,54±0,18	0,51±0,19

FIGURES

Figure 1 - Rat sciatic nerve injury and surgical reconstruction

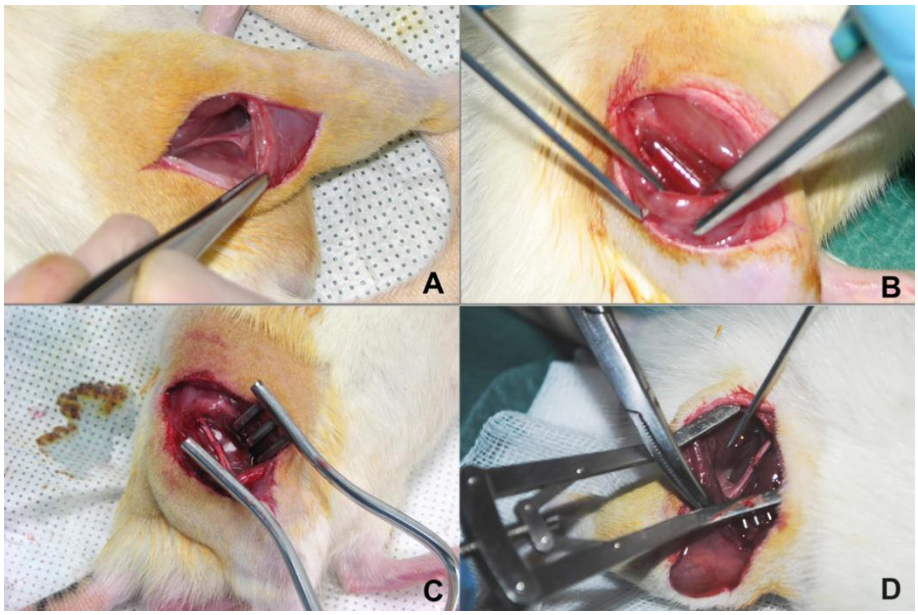
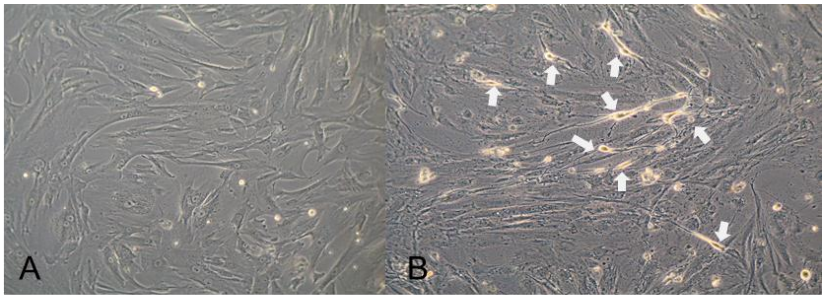


Figure 2 – Cell and membrane images



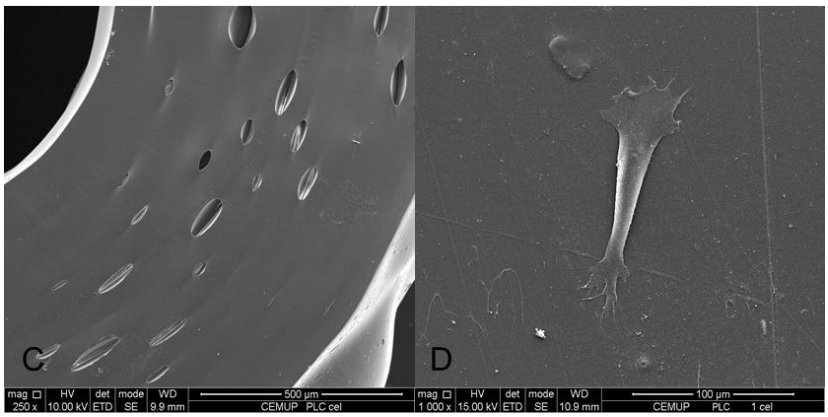


Figure 3 – Kinematic plots

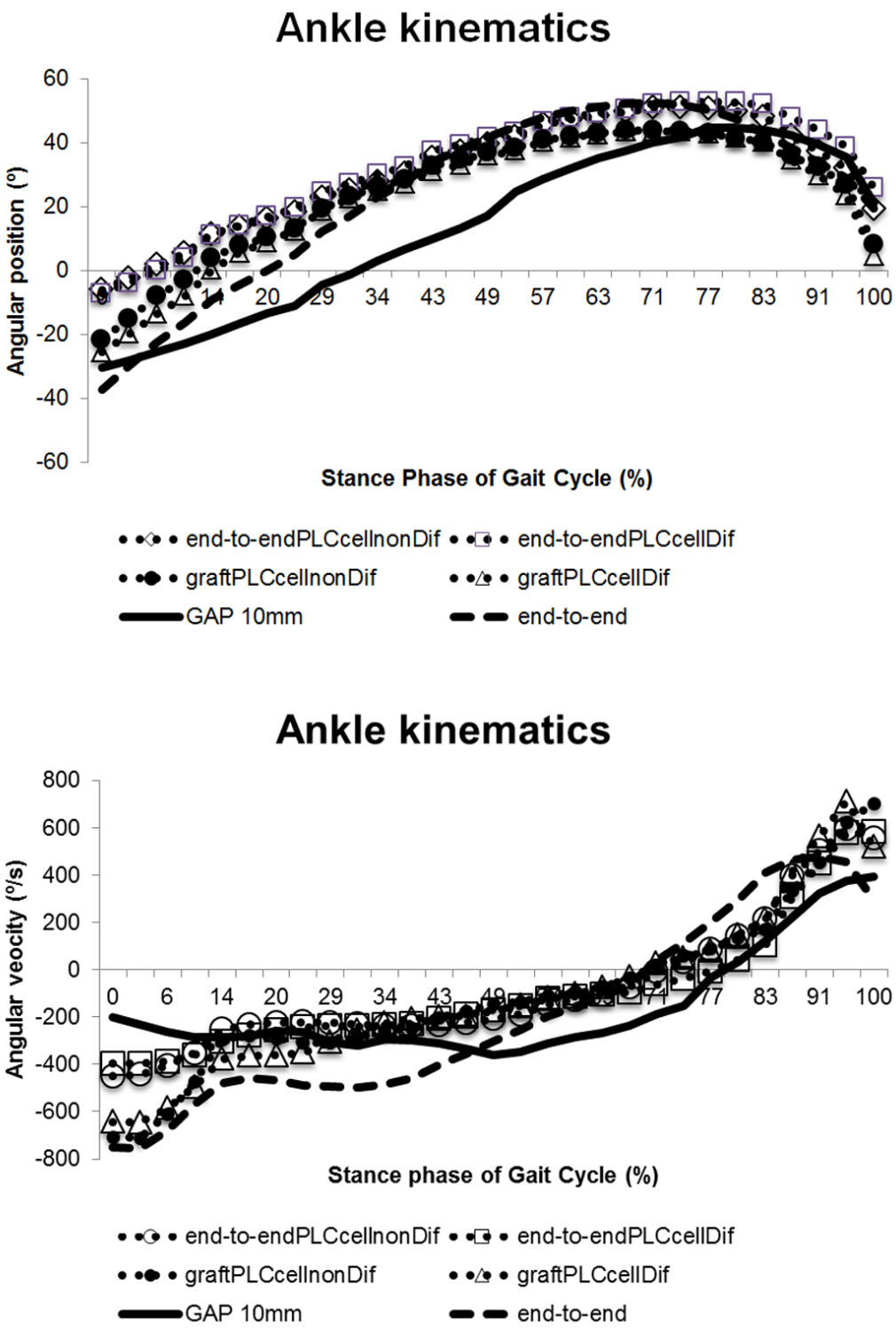


Figure 4 – Histological image

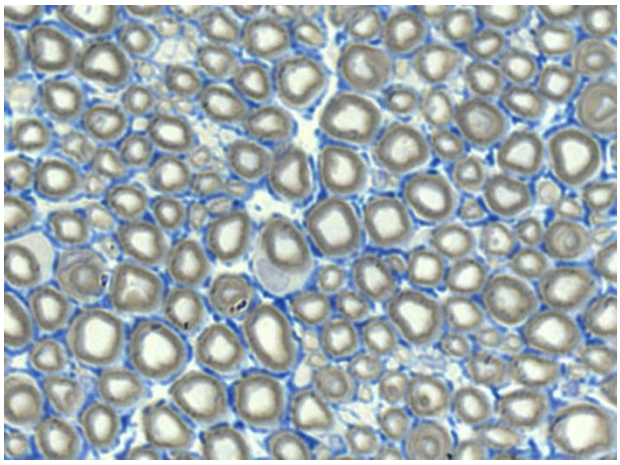
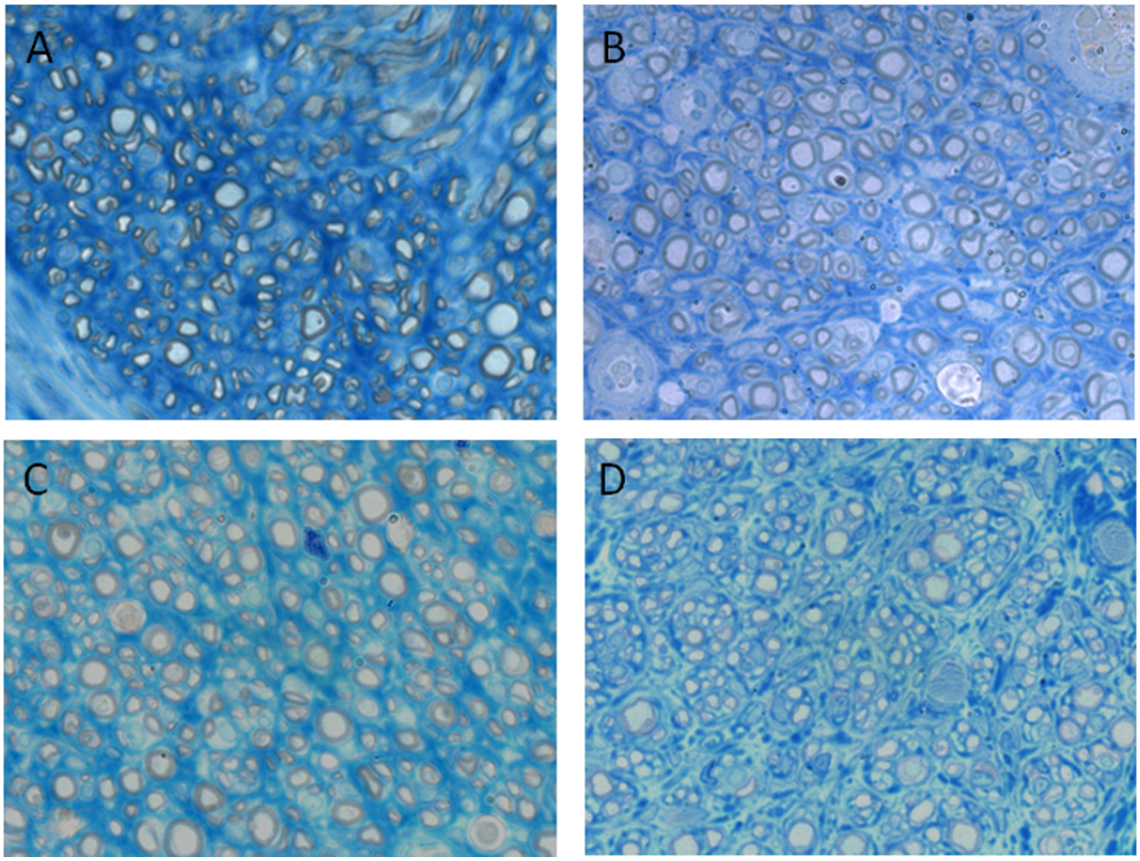


Figure 5 – Histological images



2.3
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Effects of umbilical cord tissue mesenchymal stromal cells (UCX®) on rat sciatic nerve regeneration after neurotmesis injuries.

Effects of umbilical cord tissue mesenchymal stromal cells (UCX®) on rat sciatic nerve regeneration after neurotmesis injuries

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Abstract

Background: Peripheral nerves possess the capacity of self-regeneration after traumatic injury but the extent of regeneration is often poor and may benefit from exogenous factors that enhance growth. Neonatal tissues as stem cell reservoirs offer many advantages over both embryonic and adult stem cell sources. These tissues are routinely discarded at parturition so little ethical controversy attends the procurement of the resident stem cell populations. Increasing evidence demonstrate that Mesenchymal Stromal Cells (MSCs) may play an important role in tissue regeneration through the secretion of soluble trophic factors which enhance and assist in repair by paracrine activation of surrounding cells. The use of cellular systems is a rational approach for delivering neurotrophic factors at the nerve lesion site.

Methods/Design: In the present study, the therapeutic value of a proprietary population of umbilical cord tissue-derived MSCs (UCX®) was evaluated on end-to-end rat sciatic nerve repair. After neurotmesis, the sciatic nerve was repaired by simple end-to-end suture (*End-to-End* group) and end-to-end suture in the presence of UCX®, either alone or administered with a matrix vehicle, Floseal®. Both motor and sensory functional recoveries were evaluated along the healing period using extensor postural thrust (EPT), withdrawal reflex latency (WRL) and ankle kinematics analysis. After 20 weeks animals were sacrificed and the sciatic nerves were processed for stereological analysis. The effect of UCX® administration on Wallerian degeneration in the *hyper-acute* (3 days post-operative), and *acute* (21 days postoperative) phases of healing was also evaluated through histological analysis. Motor and sensory functional recoveries were evaluated at day 7, 14 and 21 post-operative.

Discussion: Overall, the UCX® application presented positive effects in functional recovery in the hyper-acute, acute, and chronic phases of the regeneration process. The histological analysis of the *acute* phase study revealed that in the group treated with UCX® alone, the Wallerian degeneration was optimal for the subsequent process of regeneration. As expected it was not possible to observe regeneration of axons after 21 days. Instead, a little retraction of the myelin membrane was observed, which could be caused by the nerve injury. In any case, the fibre organization was higher and the extent of fibrosis was lower in the UCX®-treated group The *chronic* phase experimental groups revealed that treatment with UCX® induced an increase in number of regenerated fibres and thickening of the myelin sheet, after a healing period of 20

weeks. Finally, the UCX® application presented positive effect in functional and morphologic recovery in the acute and chronic phase of the regeneration process.

Keywords: mesenchymal stromal cells, umbilical cord tissue, neurotmesis, nerve fiber regeneration, myelinization, functional assessment, stereology, kinematics.

Introduction

A full understanding of nerve regeneration, namely complete functional achievement and organ re-innervation, after nerve injury, still remains a main goal for regenerative biology. Peripheral nerve injury has a high regenerative potential but functional recovery rarely occurs after total nerve transection. Nerve regeneration is a complex biological phenomenon. In the peripheral nervous system nerves can spontaneously regenerate without any treatment if nerve continuity is maintained (axonotmesis), whereas more severe type of injuries must be surgically treated by direct end-to-end surgical reconnection of the damaged nerve ends [1-3].

Cell transplantation has been proposed as a method of improving peripheral nerve regeneration [4, 5], including Schwann cell (SC) transplantation that can enhance axon outgrowth and survival, both *in vitro* [6] and *in vivo* [7]. Some limitations of harvesting autologous SCs, such as donor nerve sacrifice and donor site morbidity, have led to investigate other cell types that provide similar trophic support to axon regeneration [8]. Mesenchymal Stromal Cells (MSCs) have become one of the most interesting targets since they can be harvested from different sources, including neonatal tissues, without ethical concerns or limitations in cell number [9]. Besides, scientific and clinical evidence have demonstrated that MSCs have the ability to migrate to specific sites of injury or of tissue regeneration where they modulate the immune and the inflammatory responses and mobilize intrinsic cell reservoirs through a series of distinct paracrine mechanisms [10]. Furthermore, MSCs are not only capable of differentiation into tri-lineage mesenchyme cell types, such as adipocytes, chondrocytes and osteoblasts [11], but also into neuronal-like cells, including astrocytes, oligodendrocytes, microglia, neurons and neuroglial cells [12-14]. Dezawa and collaborators in 2001 showed that MSCs were also capable of differentiating into cells with Schwann cell properties [8, 15]. MSCs have been isolated from various origins, including bone marrow, skin, hair follicle, periosteum, amniotic fluid, umbilical cord blood, placenta and adipose tissue. The bone marrow represents the most commonly used tissue source for adult MSCs. Bone marrow MSCs (BM-MSCs) have been applied for cell based therapies; however, due to the limited number of BM-MSCs available, the heterologous and non-consistent nature of bone marrow preparations, the possibility of donor site morbidity, as well as decreased number of BM-MSCs along the adult life, there is a need to identify alternative and more primordial MSCs sources that would allow for safe and controlled expansion for potential allogeneic utilization. Different harvesting procedures have led to umbilical cord tissue-derived MSCs (UC-MSCs) that exhibit a neuronal phenotype

[16-19] and have potential utility in treatment of neurodegenerative diseases [20, 21], indicating the versatility of this cell source. Interestingly, these cells, which are major histocompatibility complex (MHC) class II negative, not only express both an immune-privileged and immune-modulatory phenotype, but their MHC class I expression levels can also be manipulated [18], making them a potential cell source for MSC-based therapies. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses [22].

ECBio has developed proprietary technology to consistently isolate, expand, and cryopreserve a well-characterized population of human stromal cells derived from the umbilical cord tissue, namely Wharton's jelly, trademark registered as UCX® [23]. The isolation method has been specifically designed for clinical use and it has been recently adapted according to advanced therapy medicinal product (ATMP) standards, as defined by the guideline on the minimum quality data for certification of ATMP (EMA/CAT/486831/2008/corr, 2010) (results to be published elsewhere). More recently, the UCX® paracrine activity has been demonstrated to repress T-cell activation and promote the expansion of Tregs better than BM-MSCs. Accordingly, the xenogeneic UCX® administration in an acute inflammation model showed that UCX® can reduce paw edema *in vivo* more efficiently than BM-MSCs [24]. Finally, animals treated with intra-articular (i.a.) and intra-peritoneal (i.p.) infusions of UCX® showed faster and almost full remission of local and systemic arthritis manifestations, potentially demonstrating a potent paracrine induction of tissue regeneration [24].

Cell transplantation has indeed been proposed as a method of improving peripheral nerve regeneration [4, 5] but tissue engineering of peripheral nerves may involve the application of biomaterials that will function as a vehicle for cell therapies, some of them, previously studied by the ICBAS group [1, 22, 25]. Biomaterials can support cellular systems to be able to either differentiate into neuroglial-like cells or to enhance their paracrine effects on the overall regenerative process, or can be directly involved in the regenerative process, improving the motor and sensory functional recovery. Furthermore, some biomaterials associated or not to cellular systems can also shorten the healing period thus avoiding regional muscular atrophy, which might be important in both axonotmesis and neurotmesis injuries [1, 22, 25]. Concerning peripheral nerve regeneration, it is important to identify an appropriate vehicle to the local application of the MSCs in the injury site. This vehicle must be biocompatible, support the adhesion,

expansion and survival of the cellular system, and may be a hydrogel, a tube-guide or a membrane [9, 22].

In this experimental work, the therapeutic value of UCX® was evaluated on end-to-end rat sciatic nerve repair after neurotmesis. UCX® were applied either alone or administered with a commercially available matrix vehicle, Floseal®, associated to an end-to-end suture during the chronic phase of healing in order to maintain cells as long as possible near the injury site. Floseal® is a commercial haemostatic matrix with potential application in Tissue Engineering and previously used with success by the ICBAS research group as a vehicle for Bonelike®, a modified hydroxyapatite used in bone defects [26]. Floseal® is a haemostatic sealant composed of collagen-derived particles and topical bovine-derived thrombin which has been proven to control bleeding in several medical applications, like vascular surgery, adenoidectomy, laparoscopy and partial nephrectomy. Floseal® is easily used and it can be extruded from a syringe and applied topically to the bleeding area. This haemostatic agent has the ability to acquire irregular shapes fitting the wounded site. When the Floseal® is in contact with blood, the collagen particles are hydrated and swell, restricting the blood flow. The thrombin present converts the patient fibrinogen into a fibrin polymer, originating a clot around the lesion area of the nerve [26].

Materials and methods

2.1 Ethics and regulation

Umbilical cord donations were obtained with written informed consents according to Directive 2004/23/EC which sets the standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. All the animal testing procedures were in conformity with the Directive 2010/63/EU of the European Parliament and with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC). Humane end points were followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000).

2.2. UCX® Isolation and Culture

Umbilical cords from full-term natural or caesarean births were obtained with informed consent. Cells were isolated from the umbilical cord tissue as in Santos et al. 2008 (INPI 103843, PCT/IB2008/054067, WO/2009/044379) with a number of minor alterations. Briefly, after 24 hours in contact with a saline solution containing antibiotics and anti-mycotics, umbilical cords were cleared of blood clots and transversely sectioned in 2.5cm portions. These pieces were subsequently treated with a digestion solution containing collagenase and trypsin, for 4h at 37ºC after which cells were allowed to adhere to the lower surface of a poly-D-lysine coated t-flask for 30 minutes. After this time, the liquid media was collected and centrifuged at 200g at 4ºC for 5 minutes, supernatant was collected and returned to the original t-flask and the pellet was discarded. The isolated cells were maintained in a humidified chamber at 37ºC and 7% CO_2, cells were subject to regular medium replacements and maintained until 80% of confluence. The MSC phenotype was confirmed by flow cytometry. Detection was performed with the following antibodies and their respective isotypes (all from BioLegend unless stated otherwise): PE anti-human CD105 (eBioScience); APC anti-human CD73; PE anti-human CD90; APC anti-human CD44; PerCP/Cy5.5 anti-human CD45: FITC anti-human CD34; FITC anti-human CD31; PerCP/Cy5.5 anti-human CD14; Pacific Blue anti-human CD19 and pacific-blue anti-human HLA-DR. UCX® were maintained in culture in α-MEM (Minimum Essential Medium Eagle Alpha Modification, Sigma-Aldrich) with 2mM glutamine, supplemented with 20% FBS (Heat Inactivated Fetal Bovine Serum, Invitrogen™) at 37ºC in a 5% CO_2, humid environment. For cell passage, PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich) was used prior to trypsinization to wash cells, followed by contact with TrypLE™ Select (Invitrogen™), a serum independent trypsin. TrypLE™ Select was inactivated by addition of PBS and subsequent centrifugation at 200g for 10 minutes (Sartorius, Model: 2-6). Seeding density was $1x10^4$cells/cm^2, unless indicated otherwise. Cell viability was evaluated using the trypan blue (Sigma-Aldrich) exclusion method (Figure 1A and 1B).

2.2. UCX® Tri-Lineage Differentiation

To induce adipogenic differentiation, UCX® were cultured in adipogenic differentiation medium for 3 days, consisting of α-MEM supplemented with 20% FBS, 2 mM L-glutamine, 10 µg/mL insulin (Sigma-Aldrich), 200 µM indomethacin (Sigma-Aldrich), 0.5 mM isobutylmetylxantine (Sigma-Aldrich), and 1 µM dexamethasone (Sigma-Aldrich); and subsequently 1 day in adipogenic maintenance medium, consisting of α-MEM

supplemented with 20% FBS, 2 mM L-glutamine and 10 µg/mL insulin. Medium was replaced every 3 days during 21 days, after which histochemical staining was performed. For chondrogenic differentiation, cells were grown in suspension as pellets, incubated in chondrogenic differentiation medium consisting on DMEM-LG (Sigma-Aldrich), 1% FBS, 2 mM L-glutamine, 6.25 µg/mL insulin (Sigma-Aldrich), 10 ng/mL TGF-β1 (Tebu-bio), and 50 µM ascorbate-2-phosphate (Sigma-Aldrich). Finally, to induce osteogenic differentiation, cells were incubated in osteogenic differentiation medium which composition is α-MEM, 10% FBS, 1 g/L glucose, 2 mM glutamine, 10 mM β-glycerol phosphate, 50 µg/mL ascorbate-2-phosphate, and 100 nM dexamethasone (all reagents are from Sigma-Aldrich). The medium was replaced every 3 days during 21 days and histochemical staining was performed. In adipogenic and osteogenic differentiation protocols, cells were washed and fixed with paraformaldehyde 4% for 20 min and stained with oil red O and alkaline phosphatase, respectively. For chondrogenic differentiation, cells were also fixed in paraformaldehyde 4%, dried, embedded in paraffin and cut into sections and finally stained with alcian blue. The presence of stained cells was confirmed by inverted microscopy with phase contrast (Leica, DMIL HC) (Figure 1C, 1D, 1E, 1F).

2.3. Vehicle preparation

Floseal® is a ready-to-use commercial hemostatic matrix, also applied in neurosurgery and the application kit consists of a bovine-derived gelatin matrix, a human derived thrombin component, applicator tips and several mixing accessories. The mixing accessories include a syringe with an integral female Luer connector attached, a small bowl, and a 5 ml syringe with needle attached. The mixing accessories are included to facilitate the reconstitution and mixing of the thrombin into the gelatin matrix and the applicator tips are included to facilitate the delivery of Floseal® to the site of the lesion. The gelatin matrix, manufactured by Baxter Healthcare Corporation consists of cross-linked gelatin granules and is provided sterile in a disposable syringe. The human thrombin is a sterile, freeze-dried, vapor-heated and solvent detergent-treated powder preparation made from pooled human plasma. The sterile calcium chloride solution that is used for the reconstitution of the lyophilized thrombin, the resulting thrombin solution contains 500 IU/ml of human thrombin. Floseal® is a combination of the gelatin matrix and the thrombin component, is biocompatible and resorbed with 6 to 8 weeks. The preparation of the Floseal® prior to use followed the manufacture instructions (www.floseal.com). Briefly, using the 5 ml syringe with needle attached provided in the

Thrombin component package, the 5 ml chloride solution is transferred to the vial containing the thrombin. Gently, the vial is swirled until the thrombin is dissolved. The dissolved thrombin is aspirated from the small bowl into the syringe to the indicated mark (4 ml) and the gelatin matrix granules syringe is connected to the syringe containing the thrombin solution and the gelatin matrix – thrombin solution mixture is back and forth transferred between the syringes for a total 10 passes (Figure 3A, 3B).

2.3. Microsurgical procedure

Adult male *Sasco Sprague* Dawley rats (Charles River Laboratories, Barcelona, Spain) weighing 250-300 g, were randomly divided in 5 groups of 6 animals each. All animals were housed in a temperature and humidity controlled room with 12-12 hours light / dark cycles, two animals per cage (Makrolon type 4, Tecniplast, VA, Italy), and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking in account human endpoints for animal suffering and distress. Animals were housed for two weeks before entering the experiment. For surgery, animals were placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. The surgeries were performed under an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). Under deep anesthesia (ketamine 90 mg/Kg; xylazine 12.5 mg/Kg, atropine 0.25 mg/Kg i.m.), the right sciatic nerve was exposed through a skin incision extending from the greater trochanter to the midthigh distally followed by a muscle splitting incision. After nerve mobilization, a transection injury was performed (neurotmesis) immediately above the terminal nerve ramification using straight microsurgical scissors. For the *acute phase* study, animals were randomly assigned to 3 experimental groups, in the first group (*End-to-End*), immediate cooptation with 7/0 monofilament nylon epineural sutures of the 2 transected nerve endings. In the second group (*End-to-EndUCX* group) the animals received the same treatment as the previous group but the injury local was infiltrated with UCX® suspended in culture medium and the third group, was composed of 6 healthy animals without any injury (*Control*). For these groups, animals were euthanized 21 days after the neurotmesis injury and surgery procedure. Animals were then randomly assigned to four experimental groups, and included in the *chronic phase study*. In one group (*End-to-End*), immediate cooptation with 7/0 monofilament nylon epineural sutures of the 2 transected nerve endings was performed, in a second group (*End-to-EndFlosealUCX*

group) nerve transaction was reconstructed by end-to-end suture, like in the first group, and then locally enwrapped with UCX® mixed with Floseal®. In a third group (*End-to-EndUCX* group) animals received the same treatment as the previous group but locally infiltrated with UCX® alone, suspended in culture medium. In a fourth group (*End-to-EndFloseal* group) animals received the same treatment as the previous group but locally enwrapped with Floseal® alone. Also a group of 6 animals without any injury was included (*Control*). For these groups, animals were euthanized 20 weeks after the neurotmesis injury and surgery procedure. In both studies 5000 viable UCX® cells were applied / neurotmesis lesion in a volume of 20 µl of culture medium. As functional control for both studies, a control group of animals, without any lesion (*Control* group), was considered for the histomorphometric analysis. To prevent autotomy, a deterrent substance was applied to animals' right foot [27, 28]. In order to also evaluate the effect of UCX® in the *hyper-acute phase* of the healing period, 4 more animals were euthanized 3 days after the surgical procedure for repairing the neurotmesis lesion for histological analysis: end-to-end suture (*End-to-End*), end-to-end suture covered by Floseal® (*End-to-EndFloseal*), end-to-end suture infiltrated with UCX® (*End-to-EndUCX*), and end-to-end suture with UCX® mixed with Floseal® (*End-to-EndFlosealUCX*). In this experimental group, 5000 viable UCX® cells were also applied in the neurotmesis lesion either suspended in 20 µl of culture medium or in the appropriate vehicle (Floseal®). The animals were intensively examined for signs of autotomy and contracture post-operative and none presented severe wounds, infections or contractures (Figure 2A, 2B and Figure 3C).

2.4. Functional assessment

The *chronic phase* groups (*End-to-End; End-to-EndFlosealUCX; End-to-EndUCX; End-to-EndFloseal, Control*) were tested pre-operatively (week 0), every week up to week 12 and every 2 weeks until the end of follow-up time (20 weeks). The *acute phase* group (*End-to-End*, *End-to-EndUCX,* and *Control*) were tested preoperatively (week 0), and at days 7, 14 and 21. Animals were gently handled, and tested in a quiet environment to minimize stress. No functional assessment was performed in the *hyper-acute phase* experimental group.

Evaluation of motor performance (EPT) and nociceptive function (WRL)

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively. The animals

were tested pre-operatively (week-0), at day 7, 14, and 21 (*Acute phase study*); and pre-operatively (week-0) and every week until week 12 and every 2 weeks until the end of follow-up time, 20 weeks (*Chronic phase study*). The EPT was originally proposed by Thalhammer and collaborators, in 1995 [29] as a part of the neurological recovery evaluation in the rat after sciatic nerve injury. For this test, the entire body of the rat, excepting the hind-limbs, was wrapped in a surgical towel. Supporting the animal by the thorax and lowering the affected hind-limb towards the platform of a digital balance, elicits the EPT. As the animal is lowered to the platform, it extends the hind-limb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance (model TM560; Gibertini, Milan, Italy) was recorded. The same procedure was applied to the contralateral, unaffected limb. Each EPT test was repeated 3 times and the average result was considered. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into an equation (Equation 1) to derive the functional deficit (varying between 0 and 1), as described by Koka and Hadlock, in 2001 [30].

Motor Deficit = (NEPT – EEPT) / NEPT (Equation 1)

To assess the nociceptive withdrawal reflex (WRL), the hotplate test was modified as described by Masters and collaborators [31]. The rat was wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate at 56ºC (model 35-D, IITC Life Science Instruments, Woodland Hill, CA). WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. Normal rats withdraw their paws from the hotplate within 4.3 s or less [32]. The affected limbs were tested 3 times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies were averaged to obtain a final result [33]. If there was no paw withdrawal after 12 s, the heat stimulus was removed to prevent tissue damage, and the animal was assigned the maximal WRL of 12 s [34, 35].

Kinematics analysis

Ankle kinematics was carried out prior nerve injury (week 0), and at the 20-week follow-up time and it was only performed in the *chronic phase* study. A group of 6 rats presenting a neurotmesis injury with a gap of 10 mm where no surgical or cellular therapy was performed was included for data analysis (*Gap* group). Animals walked on

a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus was adjusted to the size of the rats during the experiments. The rats' gait was video recorded at a rate of 300 Hz images per second (Casio Exilim PRO EX-F1, Japan). The camera was positioned at the track half-length where gait velocity was steady, and 1 m distant from the track obtaining a visualization field of 14 cm wide. The video images were stored in a computer hard disk for latter analysis using an appropriate software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, USA). 2-D biomechanical analysis (sagittal plan) was carried out applying a two-segment model of the ankle joint, adopted from the model firstly developed by Varejão [36]. The rats' ankle angle was determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (θ^{o}) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points were identified: initial contact (IC), Opposite Toe off (OT), and Heel Rise (HR) and toe-off (TO) [36-38] and were time normalized for 100% of step cycle. The normalized temporal parameters were averaged over all recorded trials. A total of six walking trials for each animal with stance phases lasting between 150 and 400 ms were considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) [36].

2.5. Sciatic nerve stereology and histological analysis

For the *chronic phase* study, histomorphometric analysis was performed. Nerve samples obtained from the (10-mm-long sciatic nerve segments distal to the neurotmesis site and from un-operated controls) were processed for quantitative morphometry of myelinated nerve fibers [39]. Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding was obtained following Glauerts' procedure [40]. Series of 2 µm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology was carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-dissector were adopted using a protocol previously described [41, 42]. Fiber density and total number were estimated together with fiber and axon diameter and myelin thickness. For the *acute phase study*, the animals were

euthanized 21 days after surgery and the samples' histological preparation was as described for the histomorphometric analysis. For the *hyper-acute phase study* the nerve samples were collected at day 3, fixed in 10% buffered formalin, routinely processed, dehydrated and embedded in paraffin wax. Consecutive 4µm-sections were cut, stained with haematoxylin and eosin (HE) and submitted to histological evaluation where a quality analysis was performed by a single operator.

2.6. Statistical analysis

For data regarding the functional tests, means and standard deviations (SD) were computed and reported for each time point, including pre-operatively, and each experimental group. Differences between time points and between groups were tested by two-way analysis of variance (ANOVA) using a mixed model of within- (time of recovery) and between-subjects (experimental groups) factors. In case ANOVA revealed an overall significant main effect of experimental group (between-subjects factor), pairwise comparisons between the groups was undertaken using the post hoc Tukey's HSD test. Statistical significance was accepted at a level of $p<0.05$. For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test. Statistical significance was established as $p<0.05$. All statistical procedures were performed by using the statistical package SPSS (version 14.0, SPSS, Inc) except stereological data that were analyzed using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milan, Italy). All data in this study is presented as mean ± SD. Statistical significance was accepted at $p<0.05$.

3. Results

3.1 UCX® are Mesenchymal Stromal Cells (MSCs) according to the ISCT

MSCs, as defined by the International Society for Cellular Therapy (ISCT), are cells characterized by: a) their capacity to adhere to plastic; b) positive expression of specific surface markers, namely CD44, CD73, CD90, and CD105, and negative expression of CD14, CD19, CD31, CD34, CD45 and HLA-DR. Additionally, according to the ISCT, MSCs are able to undergo tri-lineage differentiation into adipocytes, chondroblasts and osteoblasts [43]. UCX® were expanded to P5 (12.3±2.5 generations, N=8) where the culture appeared homogeneous and cells presented their typical fusiform, fibroblast-like, morphology (Figure 1C). As expected for MSC-type stromal cells, flow cytometry analysis showed that over 98% of the cells in the population were consistently positive for the cell surface markers CD44, CD73, CD90 and CD105 and less than 2% positive

for CD14, CD19, CD31, CD34, CD45 and HLA-DR (Figure 1B, 1C). In order to assay for UCX® capacity for tri-lineage differentiation, UCX® expanded to P5 were incubated with specific differentiation media as described in the methods section. Results showed that UCX® cells have the capacity for tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts (Figure 1D, 1E, 1F). Control samples on the left column are cells undergoing the same culture conditions and specific staining reactions but without addition of differentiation factors (Figure 1C). For chondrogenic differentiation, the natural tendency for UCX® to form three-dimensional aggregates has become noticeable, even without the addition of differentiaion factors (Figure 1E). In any case, chondrospheres resulting from terminal chondrogenic differentiation are consistently larger, more regularly shaped, and strongly stain positive for alcian blue (Figure 1F).

3.2. Motor deficit, Nociception function and SFI

For the *acute study* groups the percentage motor deficit increased to near maximum values in the immediate week following sciatic nerve cut and repair, remaining elevated without significant recovery during the following 3 weeks [$F(2,28) = 2.233$, $p = 0.126$]. The EPT deficit was relatively less severe in animals treated with UCX® during the 3 weeks following sciatic nerve transection and direct cooptation ($p < 0.05$) (*End-to-EndUCX* group). Immediately after the sciatic nerve cut and repair the WRL values increased to the test termination duration of 12 seconds in almost every animal, and slightly improving during the following 3 weeks. Both, the degree of acute affection in thermal and nociceptive sensitive function and the rate of its recovery were similar in both treated groups during the study period (Table 1 and Table 2).

For the *chronic study* groups, data from withdrawal reflex latency (WRL) were analyzed through two-way Anova for repeated measures, comparing groups after sciatic neurotmesis treated with UCX® (*End-to-EndUCX* group), Floseal® (*End-to-EndFloseal* group), these two treatments combined (*Endto-EndFlosealUCX* group) and an end-to-end control group (*End-to-End*). The results showed a significant interaction effect between the variables time of recovery and treatment [$F(33,220) = 3.097$; $p = 0.000$]. This effect was explained by differences in recovery of the WRL after sciatic nerve neurotmesis between the end-to-end group (*End-to-End*) and the remaining experimental groups, with the former animals showing better recovery in this test. No differences in WRL recovery were found between animals treated with Floseal® (*End-to-EndFloseal* group), MSCs (*End-to-EndUCX* group) or with combined Floseal® plus UCX® (*Endto-EndFlosealUCX* group) (Table 3 and Table 4).

3.2. Kinematics analysis

Ankle joint kinematics during gait was assessed in the experimental treated groups at the end of the 20-weeks recovery time and was compared with data from uninjured control animals. Ankle angle was compared between groups at four selected instants belonging to the stance phase and significant differences between the groups were found for each of these time points, except for TO. Post hoc pairwise comparisons showed that no differences in ankle joint angle existed between the uninjured control group and any of the sciatic-treated groups. However, differences in this parameter existed between experimental groups. At the instant of IC, the ankle joint angle was significantly different when comparing the *End-to-End* group to any of the remaining sciatic-treated groups (*End-to-EndFlosealUCX* , *End-to-EndUCX* and *End-to-EndFloseal*) ($p<0.05$). At OT, which occurs around 20% of the entire stance phase duration, differences between groups were now found between the *End-to-End* and *End-to-EndUCX* ($p=0.023$), and *End-to-EndFlosealUCX* ($p=0.005$), as well as between the two latter groups ($p=0.015$). Further differences in ankle angle at this time point were observed between the *End-to-End* group and the group treated with UCX® and Floseal® (*End-to-EndFlosealUCX*) ($p=0.038$). At the instant of HR, meaning the time during the stance phase of the rat's gait cycle when the push off action begins, significant differences in ankle joint angle existed between *End-to-EndFloseal* animals and those within the *End-to-EndFlosealUCX* group ($p=0.008$) (Figure 4).

3.3. Histological and Stereological Analysis

In the *acute phase* of the healing period (21 days after neurotmesis) the samples revealed Wallerian degeneration in varying degrees, from mild to severe, in all material submitted for histology, hindering the observation of axonal regeneration (Figure 5A and 5B). A little retraction of the myelin membrane was observed, which could be caused by the nerve injury. The fiber organization was higher and the extent of fibrosis was lower in the *End-to-EndUCX* group (Figure 5A and 5B).

In the *hyper-acute phase* (3 days after neurotmesis), the histology of the *End-to-end* sample showed disruption of the perineurium, fascicles disorganization, proliferation of Schwann cells with axonal swelling and inflammatory infiltration in the endoneurium Scarce neutrophils and multinucleated giant cells were also identified, mainly located around hair shafts, probably due to the surgical technique (Figure 6A). In turn, animals belonging to the *End-to-EndFloseal* and *End-to-EndFlosealUCX* groups developed multiple foci of dystrophic calcification, often accompanied by a foreign body

inflammatory reaction (Figure 6B and Figure 6C). In the samples from the *End-to-EndFlosealUCX* group, this finding was even more prominent and extensive, associated with deposition of large amount of fibrin and hemorrhage which destroyed the normal architecture of the nerve (Figure 6D). Mild to moderate amount of mixed inflammatory infiltrate along with elongated mesenchymal cells circumscribing the perineurium were observed in the *End-to-EndUCX* sample (Figure 6C). Such observations were indicative of an UCX® - mediated induction of Wallerian degeneration necessary for proper nerve regeneration; an assumption that turned out to be confirmed by long-term observations (Figure 7) and stereological estimates (Table 5). While light microscopy analysis was restrictive, limiting our observations to the expected smaller size of regenerated nerve fibers relative to the controls, stereological analysis disclosed a significantly ($p<0.05$) larger axon diameter and thicker myelin thickness in the *End-to-EndFlosealUCX* group when compared to either *End-to-EndUCX* or *End-to-EndFloseal* group (Table 5).

4. Discussion

Despite great progress in the fields of tissue engineering and stem cell therapy, translational and preclinical studies are required to accelerate the clinical application of alternative approaches to autologous nerve grafts for peripheral nerve repair. The peripheral nervous system is able to regenerate after traumatic injury but, most frequently, the functional outcomes following damage are poor. Nowadays, most tissue engineered nerve grafts are composed of a neural scaffold prepared with a variety of biomaterials. The introduction of active cells delivered by appropriate vehicles, could add on to the system an important biochemical cue, producing engineered nerve grafts with enhanced ability to repair more extensive peripheral nerve defects [9].

The rat is the animal model most frequently used in peripheral nerve regeneration studies because of the widespread availability of these animals as well as the distribution of their nerve trunks which is similar to humans [2]. Furthermore, the rat sciatic nerve provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and/or introduction of grafts or tube-guides [44]. Concomitantly, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type, competing to reach and re-innervate distal targets [2]. Peripheral nerve injuries are classified in two major groups, crushing injuries (axonotmesis) and complete nerve sections (neurotmesis) with or without loss of nerve tissue [45, 46]. The axonotmesis

injury is less severe and it is often used in studies concerning the physiological mechanisms of regeneration, while neurotmesis is mostly used in surgical strategy studies, such as the implementation of biomaterials associated with cellular systems [22, 25].

The crucial role of stem cells, mesenchymal stem cells (MSCs) in particular, in tissue renewal and regeneration has been well established [47]. The International Society for Cellular Therapy has established minimal criteria to define MSCs. Namely, these cells should have the ability to adhere to plastic in optimal culture conditions, should express CD105, CD73, and CD90 surface markers, should not express CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR, and should be able to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* [43].

ECBio has developed and patented an isolation method that consistently isolate a population of stem cells from the umbilical cord tissue that fulfils the International Society for Cellular Therapy (ISCT) criteria for MSCs [23]. The cellular product has been registered under the trademark UCX®. UCX® isolated with this method have proven not to be immunogenic able to induce immune tolerance in vivo by repressing T-cell activation and promoting the expansion of Tregs and in a chronic adjuvant-induced arthritis model, animals treated with UCX® showed faster remission of local and systemic arthritic manifestations [48]. More recently, the UCX® grown in aggregates that better mimic tissue environment have produced a secretome rich in trophic factors, such as HGF, TGF-β, G-CSF, VEGF-A, FGF-2, KGF and IL-6 that promote wound healing reactions, as demonstrated in vitro by vasculogenesis, motogenic and chemotactic assays; and in vivo, using a chemotaxis assay where UCX® were shown to recruit surrounding bone marrow MSCs known to be directly involved in tissue regeneration (results to be published elsewhere).

In the present study the *in vivo* application of UCX® was intended to improve the regeneration process in the rat sciatic nerve after a neurotmesis injury which was surgically reconstructed using and-to-end suture. We hypothesize that UCX® can integrate surrounding the lesion tissue and modulate the inflammatory response (including the Wallerian degeneration that occurs in the *hyper-acute phase*) though a paracrine mechanisms that involves trophic factors able to promote local vascularization and mobilization of local cells (like the Schwann cells) involved in nerve regeneration [49]. From the beginning, the risk of immune rejection in our experimental setup was neglected. MSCs derived from the Wharton's jelly, including the UCX® cells

have already been xeno-implanted before into rabbits, rats and sheep by us and other research groups, without eliciting any compromising immune rejection reaction [24, 50].
In this experimental work, it was important to evaluate the immediate Wallerian response and early nerve regeneration in the *hyper-acute* and *acute phases*, respectively, given the large importance of the Wallerian degeneration for subsequent cellular and molecular events that will promote nerve regeneration. When an axon is transected, the distal cytoskeleton disintegrates, its cell membrane disappears, and the axon fragments. The Wallerian degeneration is accompanied by macrophages entering the transected area to remove myelin and axonal debris being first detected by light microscopy 36 to 44 hours after nerve transection in mice and rats, and reaching a peak around the third week. In the *acute phase* of the healing (21 days after neurotmesis), our samples revealed Wallerian degeneration in varying degrees, from mild to severe, hindering the observation of axonal regeneration. On the other hand, nerve fiber organization was higher and the extent of fibrosis was lower when sciatic nerves were treated with UCX®, which suggests the importance of these cells in improving nerve regeneration when applied in the first days after neurotmesis by promoting an efficient Wallerian degeneration. Theoretical repercussion of these histological observations performed during the *hyper-acute* and *acute phases* were confirmed by long-term observations and stereological estimates. While light microscopy analysis was limited, allowing to observe in all experimental groups just the expected smaller size of regenerated nerve fibers with respect to the controls, stereological analysis disclosed a significantly larger axon diameter and higher myelin thickness in the *End-to-EndFlosealUCX* group in comparison to *End-to-EndUCX* and *End-to-EndFloseal* experimental groups. These histological and histomorphometric analyses performed in the *hyper-acute*, *acute* and *chronic phases* also suggest that Floseal® is an appropriate vehicle to delivery UCX® to peripheral nerve injuries.
The myelin sheath was thicker in the regenerated nerves, suggesting that UCX® might exert their positive effects on Schwann cells, the key element in Wallerian degeneration and consequent axonal regeneration. Previous results have demonstrated that the use of either undifferentiated or neuroglial-like differentiated Wharton jelly MSCs did enhance the recovery of sensory and motor function of the rat sciatic nerve in axonotmesis and neurotmesis injuries [14, 51]. Both the degree of acute affection in thermal and nociceptive sensitive function as well as the rate of its recovery in the *acute phase* in the *End-to-End* group was similar to the *End-to-EndUCX*. Concerning EPT and WRL results in the chronic phase study, no differences in recovery were

found between any of the treated groups. However, these tests are, to some extent, more dependent on the operator and therefore subject to human error. As a result, the use of different methods for an overall assessment of nerve function has long been recommended by several investigators [52]. In our lab we complement EPT and WRL techniques with a kinematics evaluation in order to reliably predict the potential therapeutic benefit of a nerve repair strategy. In this case the kinematics analysis showed differences in ankle joint angle between experimental groups. At the instant of IC, the ankle joint angle determined for animals belonging to the *End-to-End* group was significantly different from any of the remaining treated groups. At OT, which occurs around 20% of the entire stance phase duration, differences were now found between the *End-to-End* group and the groups treated with UCX® (*End-to-EndUCX* group), and UCX® administered with Floseal® (*End-to-EndFlosealUCX*), as well as between the two latter groups. At the instant of HR, meaning the time during the stance phase of the rat's gait cycle, when the push off action begins, significant differences in ankle joint angle were measured between *End-to-EndFloseal* animals and *End-to-EndFlosealUCX* animals. Despite ankle kinematics did not demonstrate clear differences between groups in terms of functional recovery, it is worthwhile noting that, 20 weeks following sciatic nerve neurotmesis, the ankle kinematics during gait stance phase was similar to that of control animals. Because it is unlikely that sciatic-injured animals can fully restore the normal ankle joint motion pattern during gait, we have to consider that even our kinematics analysis might not have sufficient sensitivity to detect small differences in gait performance between experimental groups.

Additional studies will be necessary to clarify the potential of these cells to be used in nerve and tissue regeneration. However, the results discussed herein show a promising effect of UCX® in promoting myelin production by Schwann cells in surgically reconstructed nerves after a neurotmesis injury. A new gateway it therefore opened for using these cells in neurodegenerative diseases that are typified by demyelination.

5. FIGURES AND TABLES LEGENDS

Figure 1 - UCX® characterization.

Results from Flow Cytometry analysis of cell surface markers are presented as histograms (A) and column bars (B). Isolated UCX® presented a fusiform, fibroblast-like morphology in culture (C). Multi-lineage differentiation potential of UCX® was qualitatively analyzed by histological staining methods. Adipogenic differentiation was assessed with Oil Red O staining (D); chondrogenic differentiation with Alcian Blue staining (E); and osteogenic differentiation with Alkaline Phosphatase staining (F)s. Figure is representative of n=8 umbilical cords used in these studies .

Figure 2 – Rat sciatic nerve injury and surgical reconstruction

Rat sciatic nerve with a neurotmesis injury with a gap of 10 mm (A). The neurotmesis injury was performed and regeneration initiated after reconstruction with an end-to-end suture where the coaptation was performed with 7/0 monofilament nylon epineural sutures of the 2 transected nerve endings (B).

Figure 3 – Floseal® preparation (A and B).

The 5 ml chloride solution is transferred to a vial containing thrombin. The vial is gently swirled until the thrombin is dissolved. The dissolved thrombin is aspirated from the small bowl into the syringe to the indicated mark (4 ml) and the gelatin matrix granules syringe is connected to the syringe containing the thrombin solution (A) and the gelatin matrix – thrombin solution mixture is transferred back and forth between the syringes for a total 10 passages (B). Rat sciatic nerve reconstructed with an end-to-end suture and Floseal® (*End-to-EndFloseal* group).

Figure 4 – Kinematic plots

Kinematic plots in the sagittal plane for the ankle angle (º) as it moves through the stance phase, during the transection injury study. The mean of each group is plotted (N = 6).

Figure 5 – Histological images *acute phase*

Histological analysis of the *acute phase* of the healing period (day 21 after neurotmesis). Cross section of epon-embedded rat sciatic nerve, Toluidine Blue stain, 400x. Images of Wallerian degeneration: many axons with myelin degeneration

(arrowheads) and some regenerating axon clusters (arrows). Panels (A and B) show cross sections of nerves from the *End-to-EndUCX* group.

Figure 6 – Histological images *hyper-acute phase*
Histological analysis of the *hyper-acute phase* of the healing period (day 3 after neurotmesis). Cross section of paraffin-embedded rat sciatic nerve, HE, 100x: (A) *End-to-End*: Destruction of perineurium and endoneurium and fascicles disorganization; (B) *End-to-EndFloseal*: multiple foci of calcification in the epineurium and fatty infiltration, associated with diffuse mononuclear inflammatory infiltrate; (C) *End-to-EndUCX*: mild to moderate amount of mixed inflammatory infiltrate along with elongated mesenchymal cells that circumscribe the perineurium; (D) *End-to-EndFlosealUCX*: multiple foci of calcification associated with fibrin deposition and hemorrhage, with destruction of the perineurium and endoneurium.

Figure 7 – Light micrographs images
Light micrographs of Toluidine blue-stained sciatic nerve semi-thin sections from four different experimental groups: *Control* (A) *End-to-EndFlosealUCX* (B) *End-to-EndFloseal* (C), *End-to-EndUCX* (D). Original magnification: 1,000X.

Table 1 – Withdrawal Reflex Latency results for *acute phase*
Values in seconds (s) were obtained performing the Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed preoperatively (week-0), at day 7, day 14 and day 21, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). N corresponds to the number of animals within each experimental group.

Table 2 – Extensor Postural Thrust results for *acute phase*
Values of Motor Deficit were obtained performing Extensor Postural Thrust (EPT) test. This test has been performed preoperatively (week-0), at day 7, day 14 and day 21, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). N corresponds to the number of animals within each experimental group

Table 3 – Withdrawal Reflex Latency results for 20 weeks follow-up

Values in seconds (s) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), at week-1 and every 2 weeks after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). N corresponds to the number of animals within each experimental group.

Table 4 – Extensor Postural Thrust results 20 weeks follow-up
Values of Motor Deficit were obtained performing Extensor Postural Thrust (EPT) test. This test has been performed preoperatively (week-0), at week-1 and every 2 weeks after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). N corresponds to the number of animals within each experimental group.

Table 5 – Stereological quantitative assessment image
Stereological quantitative assessment density, total number, diameter and myelin thickness of regenerated sciatic nerve fibers at week-20 after neurotmesis. Values are presented as mean ± SD

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TABLES

Table 1 – Withdrawal Reflex Latency results for *acute phase*

	Week 0	Week 1	Week 2	Week 4
End-to-EndUCX				
1B	3,65	12,00	12,00	9,63
1C	3,45	12,00	12,00	12,00
1D	3,19	12,00	10,54	7,40
1E	3,22	12,00	12,00	11,03
1F	3,41	12,00	12,00	10,97
Mean ± SD	3,44±0,22	12,00±0,00	11,71±0,65	10,21±1,78
End-to-End				
2A	3,00	12,00	4,00	4,00
2B	2,00	12,00	5,00	12,00
2C	2,00	12,00	12,00	8,00
2D	4,00	12,00	2,00	3,00
2E	2,00	12,00	6,00	2,00
2F	2,00	12,00	12,00	12,00
2G	1,00	12,00	12,00	8,00
Mean ± SD	2,29±0,95	12,0±0,00	7,57±4,31	7,00±4,12
Control				
3A	3,00	12,00	4,00	4,00
3B	2,00	12,00	5,00	12,00
3C	2,00	12,00	12,00	8,00
3D	4,00	12,00	2,00	3,00
3E	2,00	12,00	6,00	2,00
3F	2,00	12,00	12,00	12,00
3G	1,00	12,00	12,00	8,00
Mean ± SD	2,29±0,95	12,0±0,00	7,57±4,31	7,00±4,12

Table 2 – Extensor Postural Thrust results for *acute phase*

	Week 0	Week 1	Week 2	Week 4
End-to-EndUCX				
1B	0,21	0,98	0,98	0,97
1C	-0,07	0,97	0,98	0,98
1D	-0,14	0,80	0,98	0,98
1E	-0,05	0,97	0,98	0,98
1F	0,17	0,98	0,97	0,98
Mean ± SD	0,09±0,20	0,94±0,08	0,98±0,00	0,98±0,00
End-to-End				
2A	0,07	0,90	0,80	0,73
2B	0,07	0,93	0,83	0,70
2C	0,08	0,90	0,85	0,88
2D	0,07	0,90	0,91	0,84
2E	0,09	0,97	0,97	0,69
2F	0,06	0,77	0,86	0,83
2G	0,07	0,87	0,89	0,81
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08
Control				
3A	0,07	0,90	0,80	0,73
3B	0,07	0,93	0,83	0,70
3C	0,08	0,90	0,85	0,88
3D	0,07	0,90	0,91	0,84
3E	0,09	0,97	0,97	0,69
3F	0,06	0,77	0,86	0,83
3G	0,07	0,87	0,89	0,81
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08

Table 3 – Withdrawal Reflex Latency results for 20 weeks follow-up

	Week 0	Week 1	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16	Week 18	Week 20
Control												
1A	3,00	12,00	4,00	4,00	10,00	5,00	8,00	12,00	5,00	7,00	5,00	4,00
1B	2,00	12,00	5,00	12,00	5,00	2,00	10,00	2,00	4,00	2,00	2,00	3,00
1C	2,00	12,00	12,00	8,00	5,00	3,00	4,00	4,00	4,00	6,00	4,00	4,00
1D	4,00	12,00	2,00	3,00	4,00	5,00	2,00	6,00	4,00	4,00	4,00	2,00
1E	2,00	12,00	6,00	2,00	10,00	2,00	5,00	2,00	2,00	6,00	2,00	2,00
1F	2,00	12,00	12,00	12,00	12,00	3,00	3,00	3,00	4,00	4,00	2,00	2,00
1G	1,00	12,00	12,00	8,00	10,00	4,00	2,00	2,00	2,00	3,00	2,00	2,00
Mean ± SD	2,29±0,95	12,0±0,00	7,57±4,31	7,00±4,12	8,00±3,21	3,43±1,27	4,86±3,08	4,43±3,64	3,57±1,13	4,57±1,81	3,00±1,29	2,71±0,95
End-to-EndFlosealUCX												
2A	2,93	6,03	8,75	12,00	9,74	3,57	4,66	12,00	5,91	12,00	12,00	7,43
2B	4,26	12,00	5,59	4,81	12,00	3,49	4,54	12,00	12,00	6,75	12,00	8,28
2C	5,05	12,00	12,00	12,00	4,25	6,65	12,00	12,00	12,00	12,00	12,00	12,00
2D	2,75	12,00	11,52	12,00	5,66	5,38	6,61	7,62	12,00	7,62	12,00	8,08
2E	6,53	12,00	12,00	12,00	4,42	3,74	5,83	5,62	7,56	7,83	12,00	12,00
2F	4,05	9,76	8,11	8,65	4,25	2,92	12,00	12,00	12,00	11,20	12,00	11,69
Mean ± SD	4,26±1,40	10,63±2,43	9,66±2,62	10,24±2,98	6,72±3,34	4,29±1,42	7,61±0,00	10,21±2,85	10,25±2,77	9,57±2,42	12,00±0,00	9,91±2,19
End-to-EndUCX												
3A	3,41	12,00	10,05	10,13	4,10	2,71	5,97	3,85	5,10	6,79	8,32	12,00
3B	3,05	9,19	12,00	5,63	12,00	2,76	7,02	8,52	9,04	12,00	12,00	12,00
3C	6,77	12,00	4,78	6,49	5,48	3,20	4,93	9,35	6,78	12,00	6,28	5,13
3D	5,34	12,00	11,75	11,06	3,71	3,54	6,63	5,62	4,90	4,44	12,00	12,00
3E	4,67	9,76	12,00	12,00	12,00	5,32	12,00	12,00	12,00	12,00	9,16	12,00
3F	3,41	12,00	10,05	10,13	4,10	2,71	5,97	3,85	5,10	6,79	8,32	12,00
3G	3,05	9,19	12,00	5,63	12,00	2,76	7,02	8,52	9,04	12,00	12,00	12,00
Mean ± SD	4,65±1,51	10,99±1,40	10,12±3,09	9,06±2,84	7,46±4,2	3,51±1,07	7,31±2,74	7,87±3,2	7,56±2,98	9,45±3,59	9,55±2,47	10,63±3,07
End-to-EndFloseal												
4A	4,34	12,00	12,00	12,00	12,00	12,00	10,42	12,00	12,00	12,00	12,00	12,00
4B	6,01	12,00	12,00	5,11	12,00	4,99	7,37	9,49	12,00	12,00	9,63	12,00
4C	4,39	12,00	12,00	12,00	6,52	12,00	12,00	9,09	6,53	12,00	12,00	12,00
4D	3,48	12,00	12,00	12,00	8,52	12,00	12,00	12,00	8,47	9,75	12,00	12,00
4E	6,01	12,00	12,00	4,97	6,63	12,00	10,16	12,00	12,00	12,00	12,00	7,84
4F	3,25	12,00	12,00	7,20	6,54	7,11	12,00	3,93	9,93	4,71	7,17	5,47
4G	4,34	12,00	12,00	12,00	12,00	12,00	10,42	12,00	12,00	12,00	12,00	12,00
Mean ± SD	4,58±1,20	12,00±0,00	12,00±0,00	8,88±3,51	8,70±2,67	10,02±3,14	10,66±1,82	9,75±3,15	10,16±2,29	10,41±2,93	10,80±2,02	10,22±2,86
End-to-End												
5A	3,00	12,00	4,00	4,00	10,00	5,00	8,00	12,00	5,00	7,00	5,00	4,00
5B	2,00	12,00	5,00	12,00	5,00	2,00	10,00	2,00	4,00	2,00	2,00	3,00
5C	2,00	12,00	12,00	8,00	5,00	3,00	4,00	4,00	4,00	6,00	4,00	4,00
5D	4,00	12,00	2,00	3,00	4,00	5,00	2,00	6,00	4,00	4,00	4,00	2,00
5E	2,00	12,00	6,00	2,00	10,00	2,00	5,00	2,00	2,00	6,00	2,00	2,00
5F	2,00	12,00	12,00	12,00	12,00	3,00	3,00	3,00	4,00	4,00	2,00	2,00
5G	1,00	12,00	12,00	8,00	10,00	4,00	2,00	2,00	2,00	3,00	2,00	2,00
Mean ± SD	2,29±0,95	12,0±0,00	7,57±4,31	7,00±4,12	8,00±3,21	3,43±1,27	4,86±3,08	4,43±3,64	3,57±1,13	4,57±1,81	3,00±1,29	2,71±0,95

Table 4 – Extensor Postural Thrust results 20 weeks follow-up

	Week 0	Week 1	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16	Week 18	Week 20
Control												
1A	0,07	0,90	0,80	0,73	0,68	0,56	0,54	0,54	0,64	0,48	0,42	0,50
1B	0,07	0,93	0,83	0,70	0,73	0,66	0,57	0,50	0,45	0,40	0,40	0,40
1C	0,08	0,90	0,85	0,88	0,82	0,76	0,57	0,50	0,53	0,44	0,38	0,40
1D	0,07	0,90	0,91	0,84	0,82	0,72	0,57	0,39	0,55	0,38	0,34	0,40
1E	0,09	0,97	0,97	0,69	0,60	0,56	0,51	0,46	0,45	0,38	0,38	0,39
1F	0,06	0,77	0,86	0,83	0,78	0,72	0,63	0,57	0,55	0,38	0,38	0,40
1G	0,07	0,87	0,89	0,81	0,78	0,76	0,70	0,47	0,45	0,33	0,42	0,50
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08	0,74±0,08	0,68±0,09	0,58±0,06	0,49±0,06	0,52±0,07	0,40±0,05	0,39±0,03	0,43±0,05
End-to-EndFlosealUCX												
2A	-0,29	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2B	0,11	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2C	0,11	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2D	0,06	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2E	-0,12	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
2F	0,11	1,00	0,72	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
Mean ± SD	0,00±0,17	1,00±0,00	0,95±0,11	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00
End-to-EndUCX												
3A	-0,46	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3B	0,14	1,00	0,92	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3C	0,06	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3D	0,01	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3E	-0,21	1,00	0,83	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3F	-0,46	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
3G	0,14	1,00	0,92	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
Mean ± SD	-0,09±0,24	1,00±0,00	0,95±0,08	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00
End-to-EndFloseal												
4A	-0,61	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4B	-0,10	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4C	-0,52	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4D	-0,23	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4E	0,11	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4F	0,55	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
4G	-0,61	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
Mean ± SD	-0,13±0,43	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00	1,00±0,00
End-to-End												
5A	0,07	0,90	0,80	0,73	0,68	0,56	0,54	0,54	0,64	0,48	0,42	0,50
5B	0,07	0,93	0,83	0,70	0,73	0,66	0,57	0,50	0,45	0,40	0,40	0,40
5C	0,08	0,90	0,85	0,88	0,82	0,76	0,57	0,50	0,53	0,44	0,38	0,40
5D	0,07	0,90	0,91	0,84	0,82	0,72	0,57	0,39	0,55	0,38	0,34	0,40
5E	0,09	0,97	0,97	0,69	0,60	0,56	0,51	0,46	0,45	0,38	0,38	0,39
5F	0,06	0,77	0,86	0,83	0,78	0,72	0,63	0,57	0,55	0,38	0,38	0,40
5G	0,07	0,87	0,89	0,81	0,78	0,76	0,70	0,47	0,45	0,33	0,42	0,50
Mean ± SD	0,07±0,01	0,89±0,06	0,87±0,06	0,78±0,08	0,74±0,08	0,68±0,09	0,58±0,06	0,49±0,06	0,52±0,07	0,40±0,05	0,39±0,03	0,43±0,05

Table 5 – Stereological quantitative assessment image

Group	Fiber Density (N /mm2)	Fiber Number (N)	Fiber diameter (µm)	Axon diameter (µm)	Myelin thickness (µm)
Control	15,905±2,87	7,666±1,90	6,66±0,12	4,26±0,07	1,19±0,03
End-to-EndFlosealUCX	26,009±6,512	12,255±2,290	2,88±0,42	4,57±0,51	0,85±0,05
End-to-EndFloseal	23,900±4,291	10,216±2,040	2,23±0,21	3,79±0,30	0,78±0,06
End-to-EndUCX	28,821±1,202	12,925±2,985	2,42±0,16	3,89±0,22	0,77±0,06

FIGURES

Figure 1 - UCX® characterization.

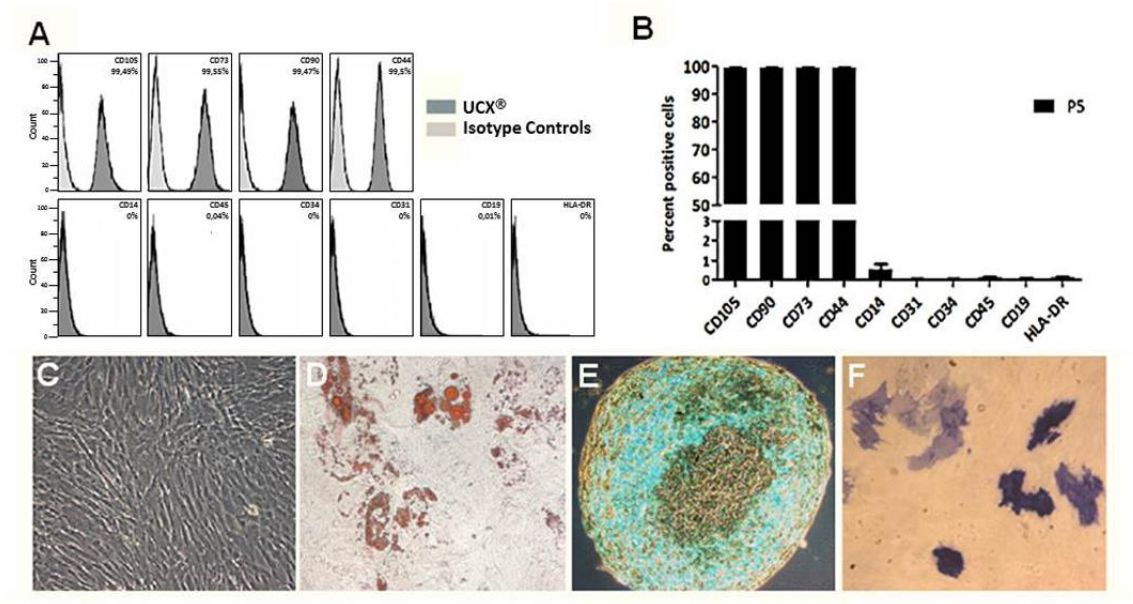


Figure 2 – Rat sciatic nerve injury and surgical reconstruction

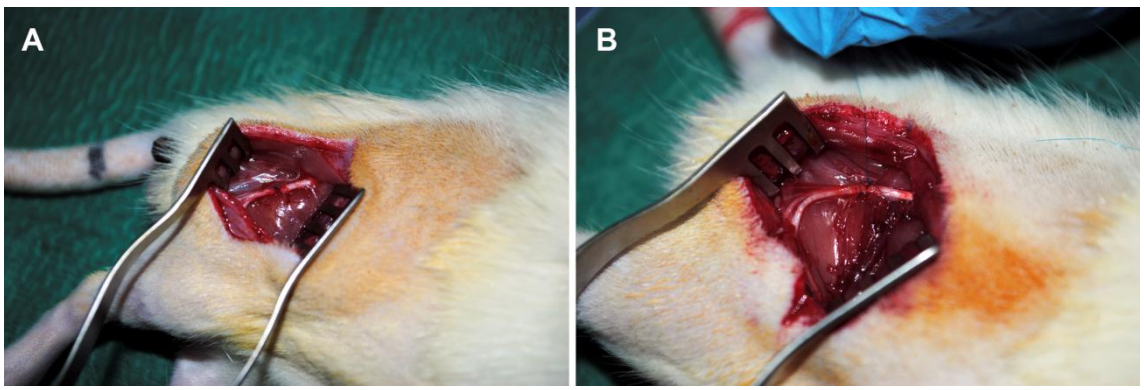


Figure 3 – Floseal® preparation (A and B).

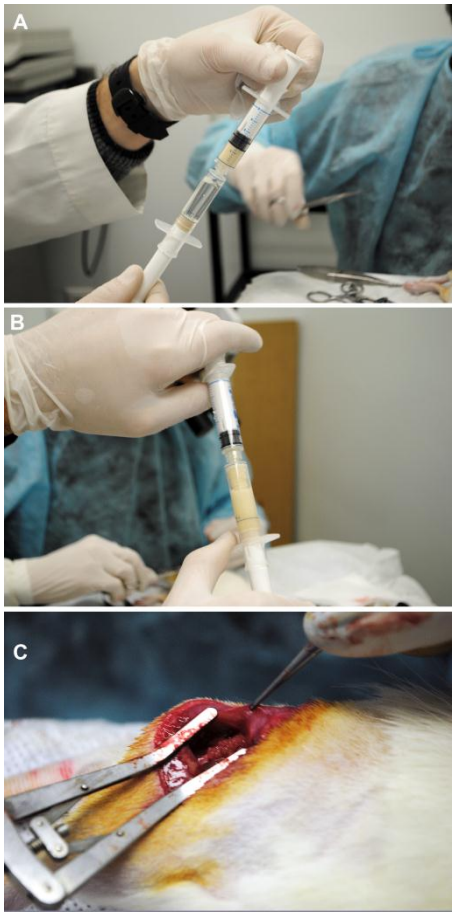


Figure 4 – Kinematic plots

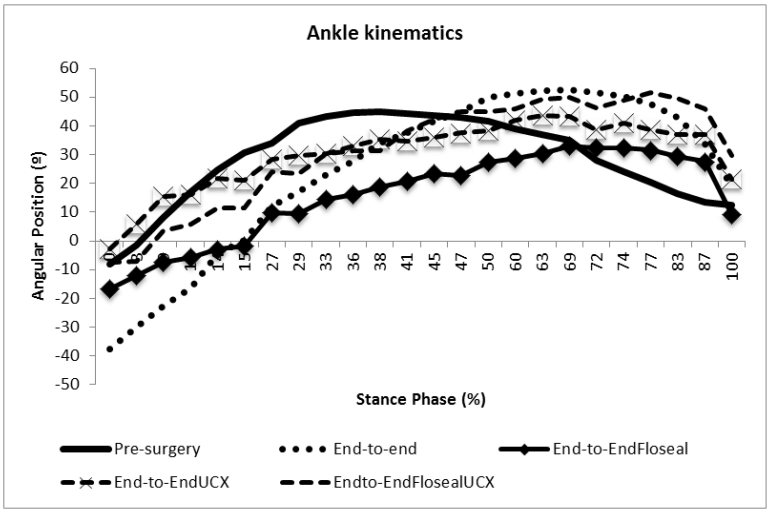


Figure 5 – Histological images *acute phase*

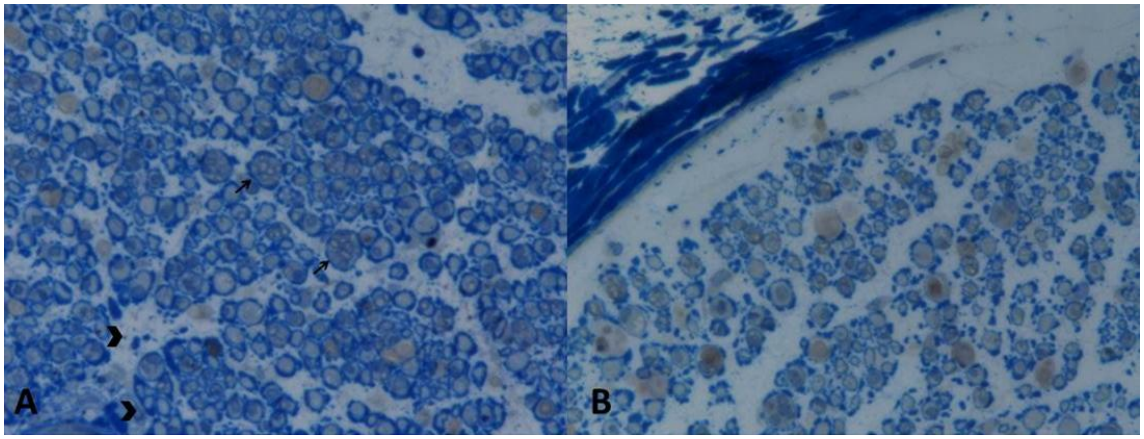


Figure 6 – Histological images *hyper-acute phase*

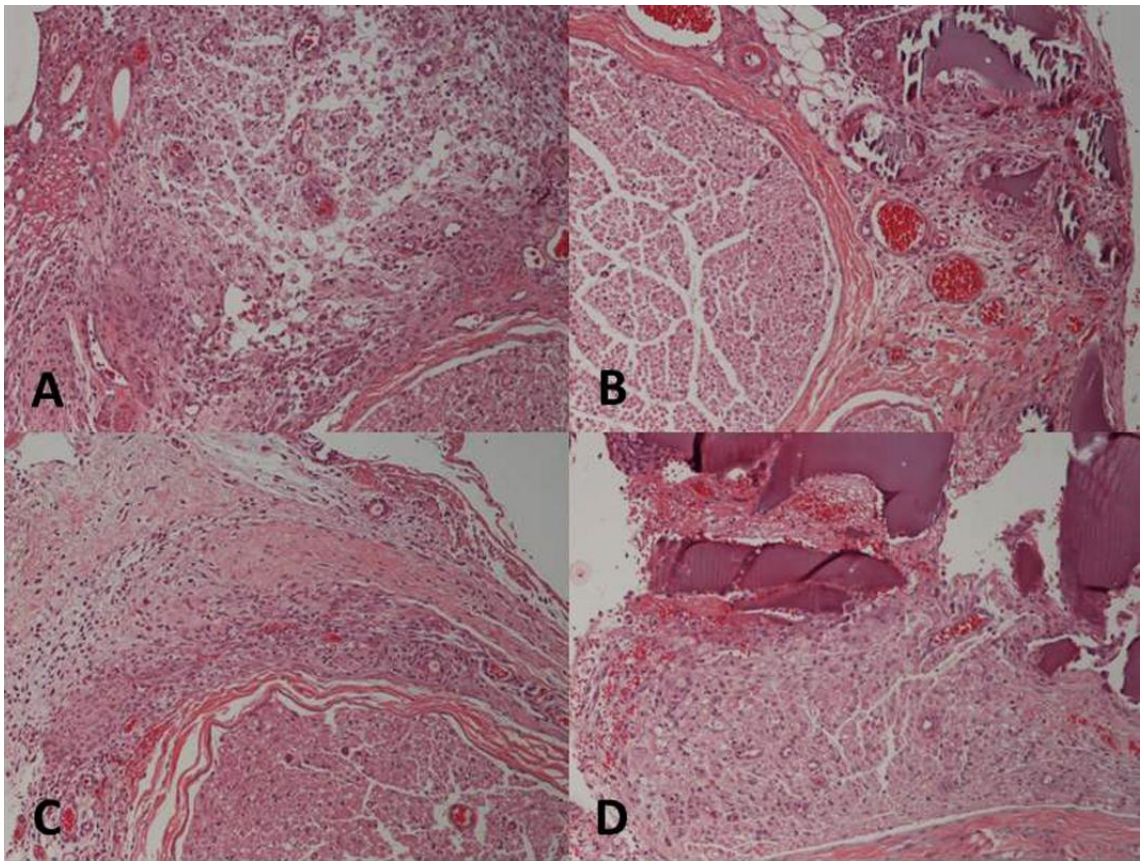
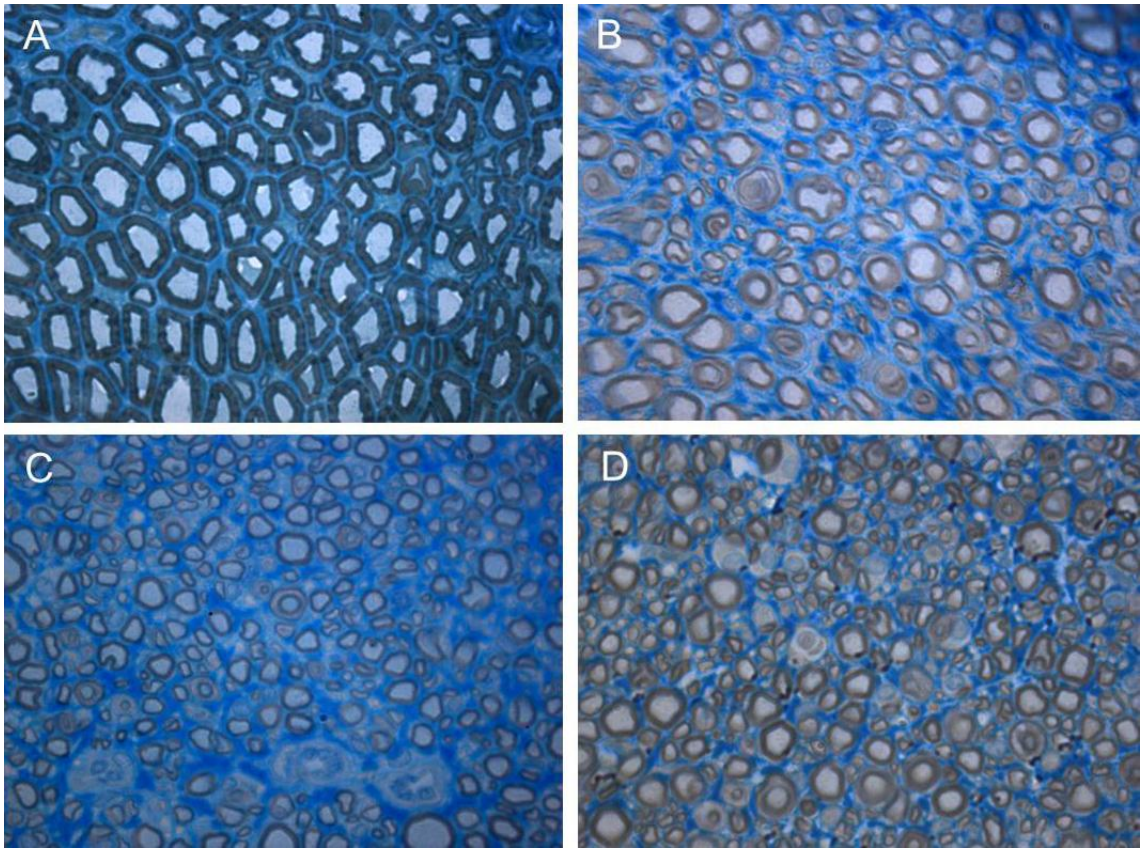


Figure 7 – Light micrographs images



2.4. Conclusions

Peripheral nerve injuries are broadly classified in two major groups, axonotmesis for crushing injuries and neurotmesis for complete nerve section with or without loss of nerve tissue (Stoll *et al.*, 1989; Chaudhry *et al.*, 1992). Neurotmesis injury is frequently used as a surgical strategy to explore the implementation of biomaterials associated with cellular systems (Luís, 2008).

As mentioned before in this chapter, association of biomaterials, to cellular systems, such as MSCs able to differentiate into neuroglial-like cells, might improve peripheral nerve regeneration, in terms of motor, sensory and histomorphometric parameters.

In the above mentioned works a biodegradable synthetic biomaterial PLC and a commercially available ready-to-use matrix vehicle, (Floseal®) were used to deliver the cellular system into the site of injury.

In conclusion, for the neurotmesis *in vivo* studies:

- The results of the 1st neurotmesis study demonstrated that enwrapment of the repaired site with a Vivosorb® membrane, with undifferentiated HMSCs or neuroglial-like cell enrichment, did not lead to any significant improvement in most of functional and stereological predictors of nerve regeneration that we assessed, with the exception of EPT which had a significantly better recovery after employment of undifferentiated HMSCs enriched membrane.
- HMSCs isolated from the Wharton's jelly of the UC delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves after neurotmesis injuries with loss of nervous tissue that enables an end-to-end suture or a grafting procedure.
- When the neurotmesis injury can be surgically reconstructed with an epineural end-to-end suture without tension or by grafting, the addition of a PLC membrane associated with a cellular system is not mandatory for a substantial improvement in functional and morphological recoveries.
- In the 2nd study it was important to evaluate the immediate Wallerian response and early nerve regeneration in the hyper-acute and acute phases, respectively, given the large importance of the Wallerian degeneration for subsequent cellular and

molecular events that will promote nerve regeneration. Nerve fibre organization was higher and the extent of fibrosis was lower when sciatic nerves were treated with MSCs, which suggests the importance of these cells in improvement of nerve regeneration when applied in the first days after the neurotmesis by promoting an efficient Wallerian degeneration.

- Histological and histomorphometric analysis performed in the *hyper-acute*, *acute* and *chronic phases* suggested that Floseal® is an appropriate vehicle to delivery UCX® cells in peripheral nerve injuries.
- The myelin sheaths were thicker in the regenerated nerves where UCX® cells were applied, suggesting that MSCs might exert their positive effects on Schwann cells, the key element in Wallerian degeneration and the following axonal regeneration.
- Additional studies will be necessary to clarify the potential use of these cells in nerve and tissue regeneration. However, the results discussed herein show a promising effect of UCX® in promoting myelin production by Schwann cells in surgically reconstructed nerves after a neurotmesis injury. A new approach therefore is open for the use of these cells in neurodegenerative diseases characterized by demyelination.

Chapter IV

Discussion and Conclusions

Discussion and conclusions

Regeneration is the physical process where remaining tissues organize themselves to replace missing or injured tissues *in vivo* (Bongso *et al.*, 2008), replacing previous function. This is the main goal of regenerative medicine.

In what concerns to peripheral nervous system, it is able to regenerate after traumatic injury, but it is still frequent to see poor functional outcome. Even with all the great advances in tissue engineering and stem cell therapy field, “prime and excellence” is still far to happen, therefore translational and pre-clinical studies are essential to speed up future clinical applications.

The choice of the experimental model has a great importance, rodents, particularly the rat and mouse, have remained during years as the chosen animal model for studies with peripheral nerve regeneration. It is relatively cheap, well studied, widespread available, as well as it’s nerve trunks distribution is similar to subhuman primates (Mackinnon *et al.*, 1985). The main issue of tissue engineering should be focused on the creation of autologous, living tissues with the potential for regeneration. Peripheral nerve regeneration must include a multidisciplinary team able to develop biomaterials, cell therapies, and to do *in vitro* and *in vivo* pre-clinical trials (Hermann *et al.*, 2004). Although sciatic nerve injuries themselves are rare in humans, the rat sciatic nerve model used in our studies provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Amado *et al.*, 2008; Liao *et al.*, 2012). Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After this kind of injury, regeneration is usually successful since axons regenerate at a steady rate along the distal nerve supported by the reactive Schwann cells (SCs) and by the preserved endoneural tubules which enhance axonal elongation and facilitate adequate reinnervation (Brohlin *et al.*, 2009). Crush injuries are appropriate to investigate the cellular and molecular mechanisms of peripheral nerve regeneration, and to assess the role of different factors in the regeneration process (Mackinnon *et al.*, 1985). Nerve crush injury is also a well-established model in experimental regeneration studies in order to investigate the impact of various pharmacological treatments and cellular therapies (Sunderland *et al.*, 2004; Amado *et al.*, 2008; van Neerven *et al.*, 2012). This injury does not implicate surgical reconstruction, but due to the required regeneration period, the neurogenic atrophy of the innervated muscles may occur. Therefore therapeutic approaches to successfully

decrease this recovery time are also important. For the reasons mentioned above, the first experimental part of this manuscript refers to this type of peripheral nerve injury.

Most tissue engineered nerve grafts are composed of a neural scaffold prepared with a variety of synthetic or natural biomaterials through well-defined fabrication techniques. Tissue engineering of peripheral nerves associating biomaterials like chitosan, PLC copolyester, collagen among others (some of them, previously studied by our group) (Amado *et al.*, 2008; Luís *et al.*, 2008b; Maurício *et al.*, 2011) to cellular systems (able to differentiate into neuroglial-like cells or modulate the inflammatory process) might improve nerve regeneration. This can be observed in terms of motor and sensory recovery, avoiding regional muscular atrophy since the healing period is shortened. The introduction of cellular systems represents a good option for tissue engineered nerve graft construction with improved ability to repair peripheral nerve injuries. SCs, MSCs, embryonic stem cells, bone marrow stromal cells are the best studied candidates. The cellular systems transplanted into the injured nerve should ideally produce growth factors or extracellular matrix (ECM) molecules, and modulate the inflammatory process, to improve nerve regeneration (Gu *et al.*, 2011; Gärtner *et al.*, 2013). There are evidences of the non-immunogenic and induce immune tolerance capacity of MSCs *in vivo* by repressing T-cell activation and promoting the expansion of Tregs and in a chronic adjuvant-induced arthritis model, animals treated with UCX® MSCs showed faster remission of local and systemic arthritic manifestations (Santos *et al.*, 2013).

Isolation of stem cells from extra-embryonic tissues, offer many advantages over both embryonic and adult stem cell sources, once these tissues are routinely discarded at birth time, so little or no ethical controversy attends the harvest of the stem cells populations. Even more, the large volume of extra-embryonic tissues increases the number of available stem cells that can be isolated (Marcus *et al.*, 2008). In contrast to the highly standardized and industrially fabricated scaffolds, the quality of stem cells varies from patient to patient, depending on the individual tissue characteristics, on the transport conditions and time of delivery of the samples from the hospital / clinic to the laboratory, on the processing and cryopreservation techniques. The MSCs isolated from extra-fetal tissues like the Wharton's jelly, have a morphologic characteristics that include a spindle shape, resembling fibroblast, that are plastic adherent (Porada *et al.*, 2010). The International Society of Cellular Therapy (ISCT) recognized the need to define MSCs. This effort was made in order to distinguish between mesenchymal stromal cells and mesenchymal stem cells. The ISCT provided a clear and resumed definition: MSCs are progenitor cells for the mesenchymal lineages. These MSCs are adherent to tissue culture

plastic (adherent cells), have a particular surface marker phenotype (express SH2, SH3, SH4, CD44, and HLA-class I, and do not express markers of the hematopoietic lineages CD34 and CD45 or HLA-class II), and have the capacity to differentiate into three mesenchymal lineages in vitro (bone, cartilage, adipocytes) (Dominici *et al.*, 2006). MSCs have been isolated from several sections of the umbilical cord. Our research group has been focused on the MSCs isolated from the Wharton's jelly and on pre-clinical studies regarding animal models for application of this cellular system to regeneration of the peripheral nervous system after axonotmesis and neurotmesis injuries. Other important experimental work related MSCs associated to bone, muscle (Pereira *et al.*, 2013) and vascular regeneration was performed alongside with these studies. These results are not referred in this thesis since it is not on its scope (unpublished data).

The aim of Ph.D. work was to explore the therapeutic value of human umbilical cord (UC) Wharton's jelly derived MSCs in *vitro* and *in vivo*, associated to different materials or vehicles on rat sciatic nerve injuries. The *in vivo* application of MSCs had the intended to improve the regeneration process in the rat sciatic nerve after axonotmesis and neurotmesis injury which was surgically reconstructed using several approaches. We hypothesize that MSCs could integrate the microenvironment surrounding the injured tissue and modulate the inflammatory response through a paracrine mechanism that encompasses trophic factors able to promote local vessel formation and mobilize local cells, like SCs involved in nerve regeneration.

***In vitro* studies**

Undifferentiated MSCs from human umbilical cord Wharton's jelly have been expanded and exhibited a normal star-like shape with a flat morphology in culture. The karyotype analysis of the MSCs cell line derived from human Wharton jelly demonstrated that this cell line has no neoplastic characteristics and was stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes (Gärtner *et al*., 2012a).

MSCs from Wharton's Jelly were differentiated into neuroglial-like cells in the presence of neurogenic culture medium during 96 hours. They became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches. The differentiation was also tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells obtained from MSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN and after 96 hours of differentiation the cells were positively stained for glial protein GFAP and for the

growth-associated protein GAP-43. All nucleus of neuroglial-like cells were also labeled with the neuron specific nuclear protein called NeuN showing that differentiation was successfully achieved (Gärtner *et al.*, 2012a). Anyway, the *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization and future experimental work concerning the use of bioreactors should be considered.

***In vivo* studies**

Several natural and synthetic biomaterials associated to cellular systems including the MSCs isolated from the umbilical cord Wharton's jelly were tested in the rat model as a therapeutic approach to treat two different sciatic nerve injuries, axonotmesis and neurotmesis. MSCs are a potential source of growth promoting signals; transplantation of these cells was expected to have a positive outcome in terms of functional and morphologic recoveries. There are two alternatives to transplant cellular systems into injured nerves. The cells may be directly injected into the neural scaffold which has been placed in the injured site or they may be pre-added on the neural scaffold via injection or co-culture (Maurício *et al.*, 2011).

In the present work the first two biomaterials described were chitosan type III developed by our research group as a hybrid of chitosan (Shirosaki *et al.*, 2005; Amado *et al.*, 2008; Simões *et al.*, 2010) and a commercially available Poly(DL-lactide-e-caprolactone) membrane, named Vivosorb®. In a group of experiments a cellular product (UCX®) derived from the umbilical cord matrix available from ECBIO (Soares *et al.*, 2008) was tested together with a commercial vehicle (Floseal®). Both of them were applied to a neurotmesis injury without loss of nervous tissue, where a surgical reconstruction using an epineural end-to-end suture was possible.

chitosan type III was developed as a hybrid of chitosan by adding GPTMS, which improves the wettability of chitosan surfaces, therefore it is expected to be more hydrophilic than the original chitosan (Shirosaki *et al.*, 2005; Amado *et al.*, 2008; Simões *et al.*, 2010). Moreover Chitosan type III was developed to be more porous, with a larger surface to volume ratio however preserving mechanical strength and the ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and this hybrid chitosan type III were previously reported as a consequence of the difference in the ability of the matrix to hold water. In fact, hybrid chitosan-based membranes may retain about twice as much biological fluid as chitosan (Chen *et al.*, 2002). The synergistic effect of a more favorable porous microstructure and

physicochemical properties (more wettable and higher water uptake level) of chitosan type III and the presence of silica ions are probably responsible for the good results in post-traumatic nerve regeneration promotion (Shirosaki *et al.*, 2005; Amado *et al.*, 2008; Simões *et al.*, 2010). In previous studies performed by our group (Amado *et al.*, 2008) *in vivo* testing demonstrated that chitosan type III improved nerve fiber regeneration in comparison to axonotmesis control. Wettability of material surfaces may be one of the key elements for protein adsorption, cell attachment and migration (Hench *et al.*, 1982).

The positive results obtained for axonal regeneration in sciatic nerves with axonotmesis injuries surrounded by chitosan type III membranes suggests that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration (Amado *et al.*, 2008; Simões *et al.*, 2010). The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation and myelinization (Amado *et al.*, 2008; Simões *et al.*, 2010; Gärtner *et al.*, 2012b).

For the tests involving chitosan type III described in this thesis, the membrane was used to deliver human MSCs from the UC of Wharton's jelly comparing this delivery approach to the direct infiltration into the injury site of MSCs in suspension. In this study the functional analysis revealed a gradual recovery of the injured hind limb during the 12-week healing period in all the experimental groups with chitosan type III and MSCs. In fact, the application of human MSCs associated or not to the chitosan membranes showed positive effects concerning the functional recovery (evaluated by EPT and WRL tests), probably due to the modulation of the inflammatory process during the Wallerian degeneration and by the production of growth factors. Once more and in agreement with previous works (Shirosaki *et al.*, 2005; Amado *et al.*, 2008; Simões *et al.*, 2010), statistically significant positive effects where observed relatively to the higher myelin thickness in the regenerated nerves enwrapped with the chitosan type III membranes alone. As expected, regenerated nerve fibers were organized in microfascicles and smaller when compared to normal control nerves. In conclusion, results of this study suggest that either enrichment of human MSCs alone or the combination of chitosan type III membrane enwrapment and human MSCs infiltration after nerve crush injury provides a slight advantage in comparison to untreated controls. On the other hand, our results confirmed that chitosan type III membranes alone may represent a very promising clinical tool in peripheral nerve reconstructive surgery.

Our PLC data showed that it does not deleteriously interfere with nerve regeneration process, as a matter of fact, the information on the efficiency of PLC membranes for promoting nerve regeneration was already provided experimentally and with patients

(Maurício *et al.*, 2011). PLC becomes hydrophilic by water uptake, which increases the permeability of the material. This is important for nutrient control and metabolite transport to the surrounding healing tissue. A few weeks after implantation, the mechanical strength gradually decreases and loss of molecular weight occurs as a result of the hydrolysis process. In approximately 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. PCL has the advantage of not creating an acidic micro-environment, which is favorable to the surrounding tissue (Luís *et al.*, 2007).

The results presented in this work (Gärtner *et al.*, 2012a) show that the use of either undifferentiated or differentiated MSCs in axonotmesis and neurotmesis lesions improved the recovery of sensory and motor function. In the cell-enriched experimental groups we observed that the myelin sheath was thicker, suggesting that MSCs certainly smear a positive effects on SCs, the key element in Wallerian degeneration and the subsequent axonal regeneration (Gärtner *et al.*, 2012a). MSCs from the Wharton's jelly with capacity to differentiate into neuroglial-like cells may be a treasure in the peripheral nervous system repair. The transplanted MSCs are able to promote local blood vessel formation and release *in locum* the neurotrophic factors (Gärtner *et al.*, 2012a; Gärtner *et al.*, 2012b). It could also be concluded that MSCs delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, after neurotmesis injuries with loss of nervous tissue.

Whenever a nerve guide is not the essential element for the therapeutic approach, and MSCs are the main component for the treatment, Floseal® will be a good choice as cell delivery vehicle. In the neurotmesis study histological and histomorphometric analyses performed in the *hyper-acute*, *acute* and *chronic phases* suggested that Floseal® was an appropriate vehicle to delivery UCX® to peripheral nerve injuries.

Additional studies will be necessary to clarify the potential of MSCs from the UC Wharton jelly (prepared using the technology developed by ECBIO or other protocol of isolation and expansion) to be used in nerve and tissue regeneration. However, the results discussed herein show a promising effect of MSCs in promoting myelin production by Schwann cells in surgically reconstructed nerves after axonotmesis and neurotmesis injuries. Therefore the use of these cells in neurodegenerative diseases typified by demyelination opens a new gateway in the field.

The MSCs isolated from the Wharton´s jelly delivered through Floseal®, PLC, and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. The

observation that in both cell-enriched experimental groups myelin sheath was thicker, suggests that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used) of the UC units from the hospital / clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved UC. It is highly recommended that the transport from the clinic to the hospital should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

Chapter V

Future Perspectives

Future Perspectives

In 2001, just a couple of months after finishing my degree in microbiology I started my scientific career with a scholarship in the Human Genetic Disease, Iron and Immune System Division of Institute for Molecular and Cell Biology (IBMC). The research topic of my scholarship was Hemochromatosis. A hereditary disease caused by the excessive absorption of iron. The accumulation of iron in organs, especially liver, heart and pancreas leads to an iron overload. The excess iron can poison these organs, leading to life-threatening conditions, with the most common being liver cirrhosis.

In 2004 with the end of the scholarship I had a proposal to start working in immunogenetic world! So, among other tasks, I had a special duty in transplantation procedure: Selection of the organ donor-recipient matching pair.

With immunogenetic, organ and bone marrow transplantation a new interest for regenerative medicine began.

Above nerve regeneration my curiosity, interest and recent scientific research knowledge are focused on stem cell therapies applied to regenerative medicine. Therefore I decided to keep on with my research work on stem cells, especially concerning the MSCs that might be isolated from several tissue sources other than the umbilical cord blood and matrix. Studying their therapeutic value on a new disease model: “liver cirrhosis”. Here by a fat of destiny was where I began this scientific adventure! For that reason, in the chapter of this thesis entitled *Future Perspectives* I decided to describe at a glance, the project that I will propose for a Pos-Doc Grant.

Abstract

The liver is a vital survival organ responsible for body homeostasis. It is well known that liver fibrosis development is strongly associated with the progression of chronic liver disease like infections by hepatitis virus B and C, alcohol abuse or nonalcoholic steatohepatitis. In last stage liver fibrosis progresses to cirrhosis, liver failure and portal hypertension which ends up requiring liver transplantation. Due to the lack donors and transplantation associated complications, it is imperative to seek alternative therapies. In the last years there has been an attempt to treat liver injury using cell therapies and tissue engineering strategies. Today, in the world over 21 million people live with chronic liver disease and 8 hundred thousand *per* year die.

The main goal of this project is to evaluate and compare the therapeutic effect *in vivo*, of allogeneic and autologous Mesenchymal Stem Cells isolated from adipose tissue and bone marrow, in an acute liver failure induced rat model (*Sasco Sprague* adult rats).

State of the Art

Liver Injury

The liver is a vital organ which comprises a wide range of functions such as metabolism, detoxification, biosynthesis of various biochemical components and storage. In adult rats the liver represents about 3% of total body weight (Martins *et al.*, 2007), with a very similar range in humans were it stands for about 2-2.5% (Grisham, 2009). Liver injury can be caused by viral infection like hepatitis B and C, drugs, excessive alcohol consumption, in addition to many genetic, metabolic and immune disorders. The most common forms of liver injury are characterized by fatty liver, steatohepatitis, liver fibrosis / cirrhosis, which might develop into hepatocellular carcinoma. Alcoholic liver disease (ALD) is one of the most common diseases responsible for these irreversible injuries. It remains a major worldwide health problem. The prognosis of individual patients with ALD depends on the degree of pathological injury, the patient's nutritional status, the presence of secondary complications of advanced disease, and the patient's capacity to eliminate destructive behaviors like alcohol consumption or inappropriate diet routine. It is well known that liver fibrosis development is strongly associated with the progression of chronic liver disease like infections by hepatitis virus B and C, alcohol abuse or nonalcoholic steatohepatitis (Friedman, 2003; Nakamura *et al.*, 2007).

Liver fibrosis is a histological change associated to parenchyma inflammation and tissue necrosis; the damage causes liver stellate cells to be over active and to increase extra cellular matrix (ECM) synthesis, more than normal amounts of collagen fiber are deposit in the extra-cellular spaces of the liver cells and the ratio of fibro-connective tissue in liver cellular tissue increases. At this stage, the liver lobular structures are intact. There is no pseudo-lobule formation. The last stage of liver fibrosis clinical progression is cirrhosis, liver failure and portal hypertension. These symptoms are serious and the clinical prognosis is poor, which implies liver transplantation. Although, fibrosis and cirrhosis are different, they are closely related. They are two distinguished pathological conditions. Cirrhosis consists of two pathological features: fibro-connective tissue hypertrophy and pseudo-lobule formation. At the cirrhosis stage, the liver's fundamental structure is deformed, and the framework of the liver begins to collapse. Therefore, reversal of the

lesions is not possible. Liver transplantation is imperative for patient's survival. New therapeutic approaches including cell therapies and tissue engineering are nowadays staring to be studied by several international research groups.

Cell Therapy

Stem cells (StC) participate in both tissue pathogenesis and the recovery process. StC are undifferentiated cells, with endless self-renewal sustained proliferation *in vitro* and multilineage differentiation capacity. They play a crucial role in many aspects of biology, from embryonic development to tissue repair and maintenance. This *in vitro* multilineage differentiation capacity has targeted these cells with extreme importance for use in tissue and cell-based therapies (Gärtner *et al.*, 2013). Stem/progenitor cells in liver tissue actively participate in the repair and functional recovery process from damage caused by alcohol and its metabolites.

MSCs comprise a rare population of multipotent progenitors capable of supporting hematopoiesis and differentiation into several lineages. Due to this ability confirmed by results of *in vitro* and *in vivo* studies, MSCs appear to be an attractive tool in regenerative medicine. The immunomodulatory function of MSCs was first reported after observation that they could evade immunosurveillance after cell transplantation (Parekkadan *et al.*, 2007).

In animal experiments of liver transplantation, hepatocytes can clonally expand, suggesting that hepatocytes are themselves functional stem cells. Certain types of severe liver injuries, especially when the proliferative capacity of hepatocytes is reduced, can activate a potential stem cell compartment in the intrahepatic biliary tree. In alcohol-induced chronic hepatitis, a close correlation has been observed between the degree of progenitor/stem cell activation and the severity of inflammation and fibrosis. Hepatic damage studies rely on the availability of animal models, although no experimental model reproduces exactly the human liver damage; they may improve our understanding on pathogenesis and future therapies.

Objectives:

The aim of this project is to evaluate and compare the therapeutic effect *in vivo*, of alogenic and autologous MSCs isolated from adipose tissue (AT) and bone marrow (BM), in an acute liver failure induced rat model.

To accomplish this goal, we aim:

1. To isolate and characterize rat (*Sasco Sprague* adult rats) MSCs from adipose tissue and bone marrow.
2. To generate an animal model (*Sasco Sprague* adult rats) for acute liver cirrhosis, administrating hepatotoxic drugs.
3. Transplant isolated MSCs by intrasplenic or intraperitoneal route in the experimental animals.
4. To evaluate and compare the anti-inflammatory response of the transplanted MSCs.
5. To analyze the MSCs secretome after they arrive to the liver tissue.

In conclusion, this project aims to evaluate the paracrine function of MSCs and their therapeutic value when used in the treatment of acute and chronic liver failure, as well as the best timing to do it and to compare allogeneic with autologous therapeutic.

Tasks description:

In the **first task of the work (Objective 1)**, we will isolation of mesenchymal stem cells from rat adipose tissue and bone marrow. Isolation will be done through the two mentioned protocols:

1st - under deep anesthesia adipose tissue from the rats flank will be collected in sterile conditions. Collection procedure will be repeated a minimum of 3 times for an average of 0.62 g of adipose tissue collected per rat.

Adipose tissue will be minced into fragments with about 4-5 mm thickness in a medium solution containing DMEM, FBS and penicillin-streptomycin.

The resulting fragments will be incubated in primary medium under standard culture conditions (37 °C with a 95% humidity atmosphere and 5% CO2).

The culture medium will be replaced every day. Cells passaged will be done using standard trypsinization method and, cell number will be counted.

MSCs will be passaged at least 4 times and then cryopreserved for future application.

Cell morphology and growth will be examined under an inverted microscope.

2nd – After euthanasia of the rat the femurs and tibias from the back limbs will be cut off and skin and muscles will be removed.

Dissected structures will be immersed in 70% isopropanol for a few seconds and transferred to DPBS.

The two ends of each bone will be cut and exposed with a scissor. A 3ml syringe attached to a 22G needle filled with DMEM will be used to flush the marrow into a 50ml tube by inserting the needle to one open end of the bone. After all the marrow is obtained, the

cells will be resuspended and passed through a 70µm cell strainer to remove the bone debris and blood aggregates.
After rinsing the cell they will be resuspended MSC medium containing DMEM, FBS and Pen-Strep. Cell suspension will be seeded and incubated under standard culture conditions for 1 to 2 weeks. Medium will be changed every 48 hours.
As soon as cells reach around 80% confluency, cell passaged will be done using standard trypsinization method. MSCs will be enriched using BD IMagnet cell separation method.
Enriched MSCs will be resuspended in culture medium and seed to be maintained or cryopreserved for future application.
Characterization of isolated MSCs will be done by flow cytometry against CD29, CD90, CD54, MHC Class I, CD45, CD106, and MHC Class II.

In the **second task of this work (Objective 2)** we will induce an acute liver damage in a rat model. Cirrhosis will be induced in rats by two methods: (i) adding phenobarbital sodium to the drinking water during 1 week, and afterwards intraperitoneal injections of 50% carbon tetrachloride ($CCl_{4)}$ (10mg/Kg body weight) twice a week during 6 weeks; (ii) administrating intraperitoneal injections of thioacetamide (TAA) (200mg/Kg body weight) twice a week during 6 weeks.
To clarify the effectiveness of the induced injury two methods will be done in parallel:
1st - There will be a first group of rats that will be sacrificed after the 6 week treatment with the hepatotoxic drugs to clarify the effectiveness of the induced injury. Cirrhosis/fibrosis will be detected by immunohistochemistry analysis for collagen type I and fibronectin of liver sections.
2nd - In parallel with histological analysis peripheral blood serum analysis will be done before sacrifice and with the remaining rats.
Peripheral blood will be collected weekly through the tail vein.
As soon as the blood clots, samples will be centrifuged. Serum will be collected for analysis of serum cholinesterase (CHE), total serum bilirubin (TSB), alkaline phosphatase (AP), creatinin (Cr), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT).

The **third task of the work (Objective 3)** will be administration of MSCs isolated from both tissue sources during 1 - 4 weeks in several trials.
MSCs will be transplanted through intraperitoneal injections. Rats will be randomly divided into several groups to receive ATMSCs with or without PBS, BMMSCs with or without

PBS and only PBS. In separate experiments rats will be sacrificed in different experimental times from 0 – 8 weeks after treatment.
To clarify and analyze the tracking of cells in the liver, transplanted cells will be labeled with a fluorescent marker to be analyzed by fluorescent in situ hybridization (FISH) in the liver tissue.

In the **fourth task of the work (Objective 4 and 5)** we will do several procedures for analysis of liver tissues obtained from the sacrificed rats.

- RNA will be extracted from liver tissue and cultured ATMSCs and BMMSCs. A total of 2 µg RNA from each source will be used to perform RT–PCR for several genes of factors that regulate the development of liver organogenesis (AFP, α-fetoprotein; CK-18, cytokeratin-18; CK-19, cytokeratin-19; HGF, hepatocyte growth factor; HNF-3β, hepatocyte nuclear factor-3β; MMP-2, matrix metalloprotease-2; PPAR-γ2, peroxisome proliferator-activated receptor- γ 2;Osteopontin; Albumin; c-Met and GAPDH). A comparative profile analysis will be done between liver tissue transplanted cells and cultured cells used for transplantation.
- Analysis by fluorescent in situ hybridization (FISH) to clarify the effectiveness of cell tracking into the cirrhotic liver tissue
- Azan Mallory histological staining or Immunohistochemistry for collagen type I and fibronectin of liver sections to define the level of liver damage reduction.

Chapter VI

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